Understanding CGTase action through the relationship between starch structure and cyclodextrin formation

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Abstract

The action of the enzyme CGTase on corn and potato starch was studied to understand the influence of starch structure and cyclodextrin (CD) production. Native and hydrolysed materials were analysed using multiple characterisation methods enabling coverage over more than four orders of magnitude in length-scale. The production of CD was greater for corn than potato but the relative abundance between different CD molecules was conserved. Structural differences were observed for all length scales investigated as a result of hydrolysis but to different extents between the starches. In corn, surface pores facilitated action of CGTase with hydrolysis generating limited changes in crystallinity and surface granule order. Hydrolysis of potato starch resulted in erosion at the granule surface, increase in surface order and overall crystallinity. Hydrolysis had minimal effect on the gelatinisation behaviour of both starches. Changes in the small-angle scattering due to CGTase confirmed the different mode of action of the enzyme between the two starches. Following hydrolysis, these changes in corn were consistent with preferential attack of the amorphous lamellae, while in potato, preferential hydrolysis of amorphous growth rings was indicated. These findings provide new insight into this industrially relevant enzyme and will assist in the

31 optimisation of CGTase-based processing conditions for the development of novel
32 products.

33 These observations were explained by differing modes of enzymatic attack between
34 starch varieties.

35 **Keywords:** cyclodextrins, CGTase, ATR-FTIR, SANS, SAXS, structure

36 Declarations of interest: none

37

1. Introduction

The enzyme cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) has been traditionally used for producing cyclic oligomers called cyclodextrins (CDs), whose complexing properties have found extensive application, including in the areas of food and pharmaceuticals (Del Valle, 2004). Specifically, CGTase hydrolyses starch (Del Valle, 2004; Szejtli, 1998) to produce CDs via an intramolecular transglycosylation reaction, although it also has other side reactions including starch hydrolysis, transference of short saccharides to other molecules (disproportionation) as well as the coupling of short saccharides and previously cleaved CDs into linear products (Qi & Zimmermann, 2005). The hydrolysis products obtained after enzymatic action are usually a mixture of α -, β - and γ -CDs comprising rings of six, seven and eight glucose monomers, respectively. These molecules possess the ability to act as molecular containers by encapsulating hydrophobic compounds within their internal cavity. As a result, substantial efforts have been undertaken not only to increase the yield of CDs but also to select and optimise the reaction conditions for producing specific CDs (Dura & Rosell, 2016; Qi, She, Endo, & Zimmermann, 2004; Zhekova, Dobrev, Stanchev, & Pishtiyski, 2009). Previous results indicated that the type and yields of CDs depend to a large extent on the type of starch used (Alves-Prado et al., 2008; Benavent-Gil & Rosell, 2017a; Blackwood & Bucke, 2000), likely due to the differences in their molecular architecture (Dona, Pages, Gilbert, & Kuchel, 2010).

More recently, CGTase has been employed to generate porous starches (Dura & Rosell, 2016) that have small rounded holes on their surface (Benavent-Gil & Rosell, 2017b), and whose number and size can be modulated with the level of CGTase applied and the type of starch substrate (Benavent-Gil & Rosell, 2017a, b). In this way, porous starches have similar characteristics to native starches but possessing enhanced absorption capacity due to their large specific surface area. Taking advantage of this greater absorption capacity, porous starches have been proposed as natural absorbents for the encapsulation of a variety of entities including drugs, microorganisms and flavours (Belingheri, Curti, Ferrillo, & Vittadini, 2012; Benavent-Gil, Rodrigo, & Rosell, 2018; Zhang et al., 2013).

Beyond the technological applications of porous starches and CDs, physiological opportunities have been identified through the joint intake of porous starches and CDs (Durá, Yokoyama, & Rosell, 2017). *In vivo* studies showed that mice fed with porous

starches combined with the released CDs augmented HDL-cholesterol levels and reduced VLDL-cholesterol levels, besides lowering total and cholesterol ester values in the liver; in addition, diets rich in porous starches combined with CDs resulted in decreased food intake offering an alternative to produce healthier foods (Durá et al., 2017). All those previous studies stated the importance of CGTase action on granular starch, either for the production of porous starches or CD release during the reaction. However, no further studies have been carried out to understand the structural changes that are undergone by starches during the CGTase reaction with the concomitant production of CDs, despite the fact that such knowledge would allow for the most appropriate selection of substrates to improve reaction yields.

Granular starch may be viewed in terms of a structure of alternating amorphous and semi-crystalline growth rings, with a gradual transition between them. The amorphous rings are formed by amylose and amylopectin with a disordered conformation, whereas the semi-crystalline rings comprise a lamellar structure of alternating crystalline and amorphous regions with a repetition distance of 9-11 nm (Blazek & Gilbert, 2011; Cameron & Donald, 1992). Any alteration in that structure can be recorded directly or indirectly using a variety of analytical techniques. X-ray diffraction (XRD) assesses and quantifies long-range crystalline order related to the lateral packing of helices.

Depending on the local organisation, several types of crystallites or polymorphs can be distinguished. In A-type polymorphs, double helices are closely packed together with a small amount of structural water. In B-type polymorphs, on the other hand, double helices are more loosely packed with proportionately more water. In contrast to the double-helical nature of the A and B polymorphs, granular starches can also exhibit so-called type V-type polymorphism arising from single amylose helices (Lopez-Rubio, Flanagan, Shrestha, Gidley, & Gilbert, 2008). Attenuated total reflectance–Fourier transform infrared (ATR-FTIR) permits the observation of short-range order at the level of the double helix (Sevenou, Hill, Farhat, & Mitchell, 2002). ATR-FTIR spectra exhibit three main modes with maximum absorbances at 1047, 1022 and 995 cm^{-1} , which are sensitive to starch structural changes. The band at 1047 (or 1045) cm^{-1} has been linked with ordered/crystalline regions, whereas the band at 1022 cm^{-1} has been associated with the amorphous regions of starch. The intensity ratios of 1047/1022 and 995/1022 cm^{-1} are widely used to characterise starch structure near the surface, since the penetration depth of the infrared beam in ATR-FTIR is about 2 μm . Neutron scattering

provides bulk structural information owing to its highly penetrating radiation source. Combined with the inherent use of large beams, the technique also provides statistically robust sampling of granules. The potential of neutron scattering techniques to food-based systems including starch was reviewed by Lopez-Rubio and Gilbert (2009) and Gilbert (2019). Specifically, small angle neutron scattering (SANS) (Blazek & Gilbert, 2011; Douth & Gilbert, 2013) allows the structural changes of starch during the course of digestion to be followed using an *in situ* digestion chamber (Blazek & Gilbert, 2010). SANS measurements were complemented by X-ray (SAXS) studies. DSC was also employed to measure changes in the thermal characteristics of gelatinisation, which is directly related to the amount of ordered carbohydrate structure within the granule (Jane et al., 1999).

With this in mind, the objective of this work was to understand the impact of CGTase-mediated hydrolysis on starch structure and its consequence on CD generation. Considering the complexity of the hierarchical structure of starch, analysis using a single analytical technique is insufficient to understand the impact of CGTase on starch structure. Consequently, the wide range of techniques was applied to assess changes covering over six orders of magnitude in length-scale from micron (SEM) down to sub-nanometre (XRD). Hence, changes in conformational order at the granule surface (SEM and ATR-FTIR), long-range order (from XRD), double helical order (from DSC) and structure at the nano-scale (SAXS and SANS) have been monitored during CGTase hydrolysis.

2. Materials and methods

2.1. Materials

Regular corn starch and potato starch from Penford Australia Pty Ltd. (Lane Cove, NSW, Australia) were used as substrates for enzymatic modification. The CGTase (EC 2.4.1.19) (Toruzyme® 3.0 L, enzyme activity declared by manufacturer 3 KNU/mL) was donated by Novozymes Australia Pvt Ltd (Sydney, Australia). All other chemicals were analytical reagent grade and used without further purification. All solutions and standards were prepared using milliQ water.

2.2. Enzymatic modification of starches by CGTase

The development and preparation of enzymatically modified porous starches is described in detail in previous publications of Benavent-Gil & Rosell (2017a, b).

Specifically, starches were treated with a concentration of 0.2 U of CGTase / g of starch for 2 hours at 50 °C (as the most efficient process to obtain porous starches), taking aliquots every 15 minutes to follow cyclodextrin (CD) production and starch changes. For every time point studied, aliquots were then centrifuged and the supernatants containing the CDs were frozen. The pellets containing undigested starch were freeze dried to ensure similar moisture content (around 5%) for further analysis.

2.3. Mass spectrometry

Electrospray ionisation mass spectra of the CDs extracted during enzymatic treatment were recorded on a 4000 QTrap AB SCIEX Mass Spectrometer. Data were acquired and processed using the Analyst v. 1.4.2 program. The capillary voltage (IS) in the positive ion mode was 5000 V. The decluttering potential was 0 V. In the full scan experiments, positive ion spectra were acquired in the 100–2000 Da mass range for samples dissolved in methanol. The concentration of CDs was calculated by using CD standards. Analysis was carried out at least in duplicate.

2.4. Scanning electron microscopy (SEM)

The granule morphologies of corn and potato starches during CGTase hydrolysis were captured using a Zeiss Ultra Plus scanning electron microscope (SEM; Carl Zeiss, Oberkochen, Germany). The samples were mounted on double sided conductive carbon tape. Approximately 3 nm of platinum was deposited onto the surfaces under vacuum to prevent charging. The obtained samples were examined at an accelerating voltage of 1 kV using the InLens secondary electron detector at 3 mm working distance and magnified 1000× times. The SEM images were further subjected to digital analysis as previously described Benavent-Gil & Rosell (2017a).

2.5. Attenuated total reflectance–Fourier transform infrared (ATR–FTIR)

ATR-FTIR spectrometric investigations were performed with a Nicolet IS10 FTIR spectrometer (Thermo Scientific, Madison, USA) and Smart iTR sampling accessory with the diamond crystal as an internal reflection element. All the ATR spectra were acquired at room temperature (22 °C) between 1200 and 800 cm⁻¹ with 32 accumulations and a spectral resolution of 4 cm⁻¹. An aliquot of sample was placed over the diamond crystal and pressed gently with a small steel cylinder. The background spectra were recorded in air (with no sample) and subtracted from the sample spectra. All the scanned spectra were baseline-corrected using "Omnic 32" software. The

baseline-corrected spectra were vector normalized by dividing each spectrum by the standard deviation of its absorbance values (with mean subtraction) (Warren, Perston, Royall, Butterworth, & Ellis, 2013). The amplitudes of absorbance for each band at 995, 1022, and 1047 cm^{-1} were recorded from the baseline-corrected spectra.

2.6.X-ray diffraction (XRD)

X-ray diffraction was carried out on a Panalytical X'Pert Pro diffractometer. The instrument was equipped with a Cu long fine focus tube, programmable incident beam divergence slit and diffracted beam scatter slit (both fixed at 0.1258°) and an X'celerator high speed detector. The samples were examined over the angular range, 2θ , of 4° to 34° with a step size of 0.03328° and a count time of 800 s per point. The Igor software package (Wavemetrics, Lake Oswego, Oregon) was used for curve fitting following the method of Lopez-Rubio, Flanagan, Gilbert, and Gidley (2008). Relative crystallinity was calculated as the ratio of the crystalline peak area to the total diffraction area.

2.7.Differential scanning calorimetry

DSC measurements were determined using a Mettler Toledo DSC 1 instrument. Sample slurries of starch and water (1:3) were weighed directly into Mettler Toledo aluminium pans. Pans were hermetically sealed and left overnight to equilibrate. Samples were heated from 30 to 120 $^\circ\text{C}$ at a rate of 10 $^\circ\text{C}/\text{min}$, using an empty aluminium can as a reference. The enthalpy of gelatinization was estimated based on the area of the main endothermic peak, expressed as Joule per gram sample (J/g) using the STARe software provided by the manufacturer. The average values were determined using duplicate measurements and normalized per gram of starch.

2.8.Small angle X-ray scattering (SAXS)

SAXS measurements were performed on a Bruker Nanostar with a rotating anode source operated at a wavelength of 1.5418 $\text{\AA}$ (Cu K_α) with a sample-to detector distance of 730 mm, giving a q range from 0.014 to 0.430 $\text{\AA}^{-1}$ where q is the magnitude of the scattering vector $= (4\pi/\lambda)\sin(\theta)$, where λ is the wavelength and 2θ is the scattering angle. The optics and sample chamber were under vacuum to minimize air scattering. The samples were presented to the X-ray beam in sealed 2 mm sealed quartz capillaries (Hilgenburg GmbH, Germany) as suspensions containing excess water above the sedimented sample and the scattering was measured for 60 min at room temperature.

The scattering patterns were reduced and radially averaged with Bruker software. A scattering background from a quartz capillary filled with buffer was subtracted after correction for sample transmission. X-ray data were placed on an absolute scale using buffer as a standard (Fan, Degen, Bendle, Grupido & Ilavsky, 2010). These operations were performed using the IRENA macro suite (Ilavsky & Jemian, 2009) within Igor (Wavemetrics). However, it is noted that scattering intensity is highly dependent on packing density and thus direct comparisons between SAXS measurements need to be conducted with caution.

2.9.Small-Angle Neutron Scattering (SANS)

SANS experiments were performed on the 40 m QUOKKA instrument at the OPAL reactor (Wood et al., 2018). SANS analysis of starch was performed according to the method of Blazek & Gilbert, (2010). Briefly, a wavelength, λ , of 5 Å and 10% wavelength resolution was used with source and sample aperture diameters of 50 and 7.5 mm, respectively. For both static and kinetic SANS measurements, sample temperature was thermostatically controlled at 50 °C. Scattering curves were plotted as absolute (SANS) intensity, I , versus q . For kinetics SANS measurements, because the sample scattering varied continuously with digestion time, a single instrument configuration was selected that includes the full q range associated with the lamellar region of native starches, namely, with a source-to-sample distance (SSD) of 3.9 m and sample-to-detector distance (SDD) of 4.1 m providing a q range of 0.015-0.31 Å⁻¹. The scattering in this configuration was recorded continuously, using event mode acquisition, throughout the 2 h of CGTase reaction; this approach enables the data to be time-binned post-experiment using a binning procedure chosen by the operator. Such an approach has been used successfully while simultaneously measuring differential scanning calorimetry (Lan et al., 2017; Pullen et al., 2014). For static SANS measurements, pre-hydrolysed (or control) samples were hydrated in phosphate buffer for at least 24 h with a concentration of 120 mg of starch in 280 µL of buffer prior to study. Three instrument configurations (SSD = 14.0 m, SDD = 12.0 m; SSD = 4.0 m, SDD = 4.0 m; and SSD = 12.0 m, SDD = 1.3 m) were used to access a q range below and above the lamellar peak from 0.0056 to 0.74 Å⁻¹. The instrumental resolution, Δq , at the main lamellar peak (located at 0.06-0.07 Å⁻¹ and discussed below) is 0.003 Å⁻¹. Enzyme dissolved in sodium phosphate buffer of the same concentration as used during the digestion was used as a background.

3. Results and discussion

3.1. Determination of the extent of starch hydrolysis and cyclodextrin production

The extent of starch hydrolysis was monitored by measuring the weight of undigested starch with respect to the original weight at specific intervals during the reaction. The kinetic curves representing the extent of hydrolysis as a function of time are shown in Figure 1A and are fitted to a first-order kinetics function as a guide only. It is worth noting that this function generally describes glucose release which is subsequently transformed into starch by a factor of 0.9.

The curves were characterized by an increase in hydrolysed starch with time. However, the amount of hydrolysed starch was significantly higher for corn starch compared to potato starch, reflecting differences in CGTase action depending on the starch source. Indeed, the extent of hydrolysis ranged from 20% for corn to 7% for potato starch after 2 h.

To assess whether starch from different botanical sources generates CDs differently, CGTase action was monitored by quantifying the levels of α -, β - and γ -CDs at specific time intervals during starch hydrolysis up to 2 h in corn and potato starches (Figure 1B and C, respectively); this duration was selected since this time has been demonstrated to be the most efficient for industrial purposes (Benavent-Gil & Rosell 2017a, b). As a result of the enzymatic activity, an overall increase in CD production as a function of hydrolysis time was noted. Under the present conditions, only β -CD production was initially (15 min incubation time) observed and, longer incubation times (30 min) were required to generate α - and γ -CDs. However, obvious differences in CD levels within samples were detected, with consistently greater values for all three CDs in corn starch compared to potato starch. Indeed, β -CD levels at the end of the hydrolysis process were 92(1) mg/100 mg for corn starch down to 49(2) mg/100 mg for potato starch, where the number in brackets refers to the standard deviation based upon replicate measurements. A similar trend was observed for α - and γ -CDs, although less pronounced. α -CD production was 60(2) (corn starch) and 30.9(1) (potato starch); while γ -CD levels were 33(2) (corn starch) and 17(2) mg / 100 mg (potato starch) i.e. the formation follows the order of β - > α - > γ -CD for both starches.

From these observations, one could surmise that corn starch is more susceptible to enzymatic attack, given that CDs obtained from corn starch hydrolysis were more abundant than from potato starch. However, care must be taken with the interpretation of these results for the analysis of starch degradation. When analysing the hydrolysis of starch by the enzyme, it should be recalled that CGTase is a multifunctional enzyme capable of catalysing different reactions (Qi & Zimmermann, 2005). The action of CGTase releases glucose, maltose and linear oligosaccharides by its hydrolysing action of starch (Benavent-Gil & Rosell, 2017b; Dura & Rosell, 2016). The oligosaccharides formed are subsequently used as substrates for the production of CDs by virtue of the CGTase cyclizing activity (Gastón, Szerman, Costa, Krymkiewicz, & Ferrarotti, 2009). A distinction must, therefore, be made here between starch hydrolysis and cyclizing activity. In this sense, the evaluation of the production of CDs only measures the cyclizing activity; it should also be noted that the increase in CDs production was not found with increasing time for all the time points studied. These observations are consistent with the multifunctional activity of CGTase. Consequently, differences in CD production could be attributed to the lower efficiency of the cyclizing activity of the enzyme when potato starch as a substrate. However, it is noteworthy that the CD ratio was identical within experimental error for both starches, viz. 0.31-0.32 : 0.50-0.51 : 0.17-0.18 for α : β : γ CD content. These results confirm findings from earlier studies (Słomińska & Sobkowiak, 1997). While the CD ratio appears to be conserved, the reaction rate differs. The latter indicates that differences in starch structure due to botanic origin influences the hydrolysis reaction(s) and suggests that the shape of the substrate determines its recognition by the enzyme (Kumar, 2010).

3.2. Morphology of starches granules during CGTase hydrolysis

Since starch hydrolysis has been often related to surface features, including granular pores and the enzyme-accessible surface area, modification of starch granules was followed by SEM during enzymatic hydrolysis. Micrographs of the starch granules, before and after the enzymatic hydrolysis are shown in Figure 2. The observed variations in granule shape and area among starches were similar to those previously reported (Benavent-Gil & Rosell, 2017a; Schirmer, Höchstötter, Jekle, Arendt, & Becker, 2013; Blazek & Gilbert, 2010). Corn starch (Figure 2A,B) exhibited a monomodal particle size distribution, with a predominant population of granules with an average area of around 120 (30) μm^2 . Granules from potato (Figure 2E,F), on the

other hand, displayed a bimodal particle size distribution. The largest granule fraction was ellipsoidal in shape with average area of 1100 (200) μm^2 , and the smallest fraction was approximately spherical in shape with an average area of 320 (80) μm^2 . These native granular morphologies remained intact as a function of hydrolysis time. The variation in surface features with hydrolysis time is shown in Figure 2C, D, and G, H. SEM micrographs also revealed variations in terms of surface features depending on starch source. For instance, typical pores described in corn starch (Fannon, Hauber, & BeMiller, 1992) were observed to be distributed all over the native starch surface (Figure 2B). Meanwhile, small abrasions on the native potato granular surface were observed (Figure 2F), likely due to the extraction and refining process (Schirmer et al., 2013). To quantitatively establish possible correlations associated with enzymatic attack and CD production, the size and frequency of surface features (related to the abundance of pores or surface abrasions per granule) were measured (Figure 3). The size distribution of surface features was significantly affected by starch source and hydrolysis time while hydrolysis time prompted a significant change in the frequency of surface features for both starches. A steady increase in pore frequency, without affecting pore size, with hydrolysis time was observed for corn starch. In contrast, potato starch granules suffered both an increase in the size and frequency of surface abrasions. Dhital, Shrestha, and Gidley (2010) stated that the presence of pores facilitates the penetration of the enzyme into the starch granule, resulting in an "inside-out" pattern of hydrolysis. Assuming that the pores observed in native corn starch fulfilled that function, no major surface modifications of the starch should be expected to occur in the initial times of hydrolysis. Between native potato starch and the first time point studied, however, a large difference of surface abrasion size was noticed. In this sense, Dura, Błaszczak, and Rosell, (2014) reported an "outside in" hydrolysis pattern due to the enzymatic attack at the most susceptible region of the starch granule surface. It is apparent that the mode of action of CGTase differs between corn and potato, with hydrolysis yielding greater changes at the granule surface in potato compared to corn starch under the same conditions.

3.3.Changes in the ATR-FTIR spectrum as a result of CGTase action

ATR-FTIR was used to evaluate changes in conformational order at the granule surface (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). The region of the spectra in the range of 1200-800 cm^{-1} is recognised as the polymer conformational fingerprint (van

Soest, Tournois, de Wit, & Vliegenthart, 1995). The relevant region for corn and potato starches during hydrolysis is presented in detail in Figures 4A and B, respectively. Both spectra exhibited the three main modes with maximum absorbances at 1047, 1022 and 995 cm^{-1} , which are sensitive to starch structural changes (Lopez-Rubio et al., 2008). Enzymatic hydrolysis did not significantly alter the ATR-FTIR spectrum of corn starch. In contrast, enzymatic hydrolysis time impact the potato spectrum at the three modes. The bands at 995 cm^{-1} and 1022 cm^{-1} have been associated with the ordered and amorphous regions of starch (van Soest et al., 1995; Warren et al., 2013). In particular, the intensity ratios of 995/1022 cm^{-1} are often employed as a useful indicator of the proportion of ordered to disordered α -glucan chains at the granule surface. The ratio of absorbance intensities at 995/1022 cm^{-1} plotted as a function of temperature for corn and potato starches are depicted in Figure 4C. The intensity ratio of 995/1022 cm^{-1} for corn starch remained unchanged during most of the hydrolysis process. However, at the end of the process, a slight increase in intensity was observed for corn as well as for potato starch. During the middle of the process (45 min digestion), this trend changed sharply to generate a marked increase in intensity for potato. Enzymatic hydrolysis in potato resulted in increased molecular order and helical organization. These results are consistent with SEM observations where greater surface changes were observed in potato starch. As mentioned earlier, an "inside-out" pattern of hydrolysis of corn starch granules could be facilitated by the presence of surface pores (Blazek & Gilbert, 2010); hence, the main structural changes may not occur at the surface and thus less extensive structural changes would be expected for corn starch. In contrast, the "outside in" pattern of hydrolysis in the potato starch granules resulted in an enzymatic attack at the weak points of the granule surface (Dura et al., 2014). Also, Li, Vasanathan, Hoover and Rossnagel (2004) reported that enzymes preferentially hydrolyse less organized areas, with the result that more organized regions accumulate (Yang et al., 2019).

3.4. Impact of CGTase action on crystalline structure

Changes in long-range order were analysed by studying the XRD patterns of hydrolysed starches at different time points (data shown in the supplementary material, Figure S1). It is well known that starches from cereals and tubers differ in their XRD patterns. A typical A-type crystalline diffraction pattern has been linked to cereal starches, while B-type crystallinity is usually found in tuber starches and high amylose starches (Buléon, Colonna, Planchot, & Ball, 1998; Hoover, 2001; Pérez & Bertoft, 2010; Zobel, 1988).

Figure 5 shows clear differences in XRD patterns between corn (Figure 5A) and potato starch (Figure 5B). Native corn starch granules exhibited reflections from the A-type polymorph with the main peaks at $\sim 15^\circ$, 20° and 23° 2θ and doublet at 17 - 18° (Figure 5A). Native potato starch revealed the B-type polymorph with the principal reflection at 17° 2θ and doublet at 23 - 24° (Figure 5B). The modified crystallinity in response to CGTase action differed depending on the starch source. The initial A-type polymorph of the native corn starch did not change extensively with only slight differences in peak height at 2θ 20° as a function of hydrolysis time (Figure 5A). In contrast, potato starch exhibited visible changes with consequent increase in crystallinity as hydrolysis progresses. Specifically, the B-type crystallinity remained, but the peaks at 23 - 24° are better resolved while another three diffraction peaks at $2\theta \sim 6$, 15 , and 20° appear, consistent with the gradual formation of the V-type polymorph (Figure 5B).

The crystallinity of starch powders was quantified according to the method described by Lopez-Rubio et al. (2008). In Figure 5C, D and E, F, an example of the fitting with 13 reflections chosen from the A- and B-type polymorphs are presented. In addition, the peak position, peak area, and crystallinity obtained after fitting are listed in supplementary materials (Table S1 and S2 for corn and potato starch, respectively). The crystallinity calculated from the XRD patterns was $\sim 37\%$ and $\sim 28\%$ for native corn and potato starches, respectively. During the hydrolysis process, there are insignificant differences observed for corn starch indicating CGTase has limited effect on starch crystallinity. In contrast, a substantial increase in crystallinity was observed during the potato starch hydrolysis with values ranged from $\sim 24\%$ (at the beginning of the process) to $\sim 40\%$ (at the end of the process). It should be noted that the rise in crystallinity after 60 minutes is associated with the increase in crystallinity values arising from the V-type polymorph.

3.5.DSC

DSC data from the two starches as a function of hydrolysis time are summarised in Table 1. Starch transition temperatures and enthalpies varied depending on starch source, which may be attributed to the differences in the degree of crystallinity (Krueger, Walker, Knutson & Inglett, 1987). In the native materials, the gelatinisation temperature for corn was greater than potato starch. In this regard, Barichello, Yada, Coffin, & Stanley, (1990) reported that high transition temperatures result from a high

degree of crystallinity, as it provides structural stability to the granule, which in turn, is more resistant to gelatinisation. These results suggest a greater degree of crystallinity in corn starch compared to potato, which is in accordance with XRD analysis. In contrast, corn starch granules exhibited a lower ΔH value than potato starch granules. Similar results have been previously described (Singh, Singh, Kaur, Sodhi & Gill, 2003). In this sense, starch enthalpy increases with increasing amylopectin branch chain-length (Jane, et al., 1999; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). It has been reported that A-type starches contain shorter amylopectin chains, while longer amylopectin branches exist in B-type starches (Perry & Donald, 2000). According to Cai et al. (2015), the amylopectin long branch-chains lead to a superior crystalline structure since they can form long helices, strengthen hydrogen bonds between chains and span the entire crystalline region (Lopez-Rubio et al., 2008). Conversely, the short branch-chains form inferior crystalline structures due to their location within a single crystalline cluster, producing short or weak double helices.

As hydrolysis proceeds, no significant changes in the transition temperatures and enthalpy for corn starch were apparent; this is perhaps reasonable as only 20% digestion has occurred even after 2 h (Dura & Rosell, 2016). However, for potato starch, there was a rapid decrease in transition temperatures and enthalpy in the first 15 minutes, despite only 7% hydrolysis occurring over the entire time period studied.

3.6.Static SAXS and SANS

SAXS and SANS were conducted to examine the nanoscale lamellar structural changes of hydrolysed corn and potato starches by CGTase enzyme following treatments as described above. Two classes of experiment were performed: (i) static – where measurements were conducted on initial and final materials and (ii) kinetic – performed with SANS using a previous method (Blazek & Gilbert, 2010) – in which measurements were performed *in situ* throughout the hydrolysis reaction; in this case, the scattering includes a contribution from the products of the CGTase reaction which occur primarily at high q .

Static SAXS and SANS patterns for corn and potato are shown in Figures 6A and 6B. Both SAXS and SANS curves revealed intense scattering at low q , followed by a rapid decrease at larger scattering angles and a scattering peak at a q -value of 0.06–0.07 $\text{\AA}^{-1}$, corresponding to a 9–10 nm semi-crystalline lamellar structure (Blazek & Gilbert,

2011). A peak at q ca. $0.4 \text{ \AA}^{-1}$ was also observable in potato starch consistent with the B-type lateral interhelix repeat (Sarko & Wu, 1978).

Following hydrolysis for corn (Figure 6A) and potato (Figure 6B), both SAXS and SANS exhibited an increase in scattering intensity at low q . In corn, the lamellar peak increased slightly in SAXS and SANS. In potato, there was an apparent increase in lamellar peak intensity but no change in the SANS. As discussed above SAXS packing density variations and their influence on relative intensities cannot be excluded. Control static SAXS and SANS measurements were made for potato starch in the absence of enzyme (Figure S3) and increases in low q scattering were observed in both SAXS and SANS indicating that such low q changes were not exclusively caused by hydrolysis; indeed, the inflection point observed was comparable to the dimensions reported for putative blocklets proposed to be related to dimensions of an amylopectin molecule (Doutch et al., 2013; Tang, Mitsunaga, & Kawamura, 2006). Such changes are not unexpected as starch granules are known to swell during hydration; a similar effect was reported by Yang et al., 2019 in corn starch where it was attributed to the amorphous water uptake due to granule swelling. The latter work is focussed on corn where, after 24 h of thermal treatment at 50°C , similar low q features were observed as found here for potato. No such changes were observed in our study for corn, either for static SAXS, static SANS nor kinetic SANS (Figure S4) where samples were studied up to a maximum time of 2 h.

3.7. Kinetic SANS

Kinetic SANS was performed for corn and potato samples in the presence and absence of CGTase. The advantage of kinetic SANS is that (i) measurements can be performed simultaneously with the digestion process at 50°C and (ii) no freeze-drying or other pre-treatments are necessary. It is also important to note that in these *in-situ* SANS measurements, as hydrolysis proceeds, the quantity of starch being measured decreases with time while the simultaneous presence of various hydrolysis products increases; however, in the static SAXS and SANS experiments, approximately similar quantities of hydrolysed starch samples, that have been previously extracted, freeze-dried and subsequently re-hydrated, are studied.

Kinetic SANS data for corn and potato starch are shown in the insets to Figure 6 while the total detector count rate versus time is shown in Figure 7 for the first hour of hydrolysis highlighting the most dramatic changes. Here, data have been binned into 2

minute intervals, possible using event-mode data acquisition. The scattering intensity in corn starch gradually increased throughout hydrolysis (Figure 7), occurring both at the lamellar peak and at lower q (Figure 6A). However, the hydrolysis in potato starch was quite different exhibiting a rapid increase at the earliest times (Figure 7). Overall these increases were less dramatic in potato than for corn with only small increases occurring at low q and lamellar peak. To verify that such changes were due to hydrolysis, kinetic SANS measurements of starches in the absence of enzyme are shown in Figure S4 where no marked changes were observed. It can be deduced therefore that, within this region of q , the behaviour in corn and potato were different: corn exhibited increases in low q and peak intensity while potato exhibited only minimal changes in low q and peak intensity. It is noted, however, that one of the origins of greater differences in corn was the more extensive hydrolysis compared to potato (20% versus 7%). The only observed changes in lamellar peak intensity for potato were observed for SAXS where sample packing density effects may be playing a role. No such effects were observed in SANS where large samples, either measured in a tumbler or flow-through cell, and packing density effects can be excluded. In addition, changes in low q behaviour could partly be attributed to granule swelling effects.

3.8. Interpreting SANS data

It is already apparent from the complementary techniques employed as well as changes in SAXS and SANS behaviour that there were marked differences in the mode of action of CGTase on corn and potato. These can be evaluated further through consideration of a paracrystalline model (Cameron & Donald, 1992) in which the starch granule can be described as a finite stack of semi-crystalline lamellae embedded in a background of amorphous growth rings. The scattering intensity is determined by the scattering length density (SLD) difference between the background material (growth ring) and the amorphous material, $\Delta\rho_b$, equal to $\rho_b - \rho_a$, and the SLD difference between the crystalline and the amorphous lamellar regions, $\Delta\rho_c$, equal to $\rho_c - \rho_a$. One of the advantages of SANS is the ability to vary the solvent contrast to manipulate the extent to which different regions in a structure can scatter neutrons. In this way, absolute values of the regional densities and corresponding SLDs have been deduced from contrast variation SANS.

Donald, Kato, Perry and Waigh (2001) and Perry and Donald (2000) showed that waxy corn, regular corn, tapioca, and rice have similar “molecular density” (or partial

physical density) of starch and water in the three regions. Potato was shown to have a substantially lower amorphous background compared with the cereal starches (crystalline lamellae ca. 1.2 g/cm³, amorphous lamellae ca. 0.5 g/cm³ for both A and B starches, and amorphous background ca. 0.7 g/cm³ for cereal starches and ca. 0.5 g/cm³ for potato). One may convert these molecular densities to volume fractions by dividing them by physical densities (1.00 g/cm³ for water, 1.72 g/cm³ for starch in crystalline lamellae and 1.59 g/cm³ for starch in amorphous lamellae and background regions) to calculate the neutron SLDs for each of the three regions (Blazek & Gilbert, 2010). The three regions - background, crystalline and amorphous – are denoted in Table 2 as B, C and A respectively (not to be confused with A- and B-type crystallinity which is a property of the starch unit cell symmetry). Thus, using neutron SLD values for crystalline starch of $2.01 \times 10^{-6} \text{ \AA}^{-2}$, for amorphous starch $1.86 \times 10^{-6} \text{ \AA}^{-2}$ and for water - $0.56 \times 10^{-6} \text{ \AA}^{-2}$, one can calculate that for corn starch, the total crystalline lamella SLD is $1.26 \times 10^{-6} \text{ \AA}^{-2}$, amorphous lamella is $0.10 \times 10^{-6} \text{ \AA}^{-2}$, and the amorphous background is $0.403 \times 10^{-6} \text{ \AA}^{-2}$. For potato, the crystalline lamella SLD is $1.06 \times 10^{-6} \text{ \AA}^{-2}$, amorphous lamella is $0.193 \times 10^{-6} \text{ \AA}^{-2}$ and for amorphous background is $0.147 \times 10^{-6} \text{ \AA}^{-2}$.

It is then possible to evaluate the effect on the SANS when specific regions of the starch granule undergo hydrolysis. Previously, Blazek & Gilbert (2010) calculated the effect of different starches hydrolysis on the scattering curve arising from preferential digestion of each of the three regions of the starch granule, namely the crystalline region of the semi-crystalline lamellae, the amorphous region of the semi-crystalline lamellae or the amorphous growth rings. In their study, they observed that the action of α -amylase and amyloglucosidase resulted in a decrease of lamellar peak intensity (more significant for A-type than for B-type starches) and a general increase in low-q scattering after 18 h of digestion. They found that (i) digestion of amorphous background (growth ring) leads to the increase in low-q scattering and does not affect the intensity of the lamellar peak; (ii) digestion of amorphous lamellae leads to the increase in the intensity of the lamellar peak and low-q scattering. Here, no reductions in low q nor peak intensity were observed; indeed, the opposite occurred despite the shorter period of hydrolysis of 2 h. The same approach to that used previously was applied here to ascertain the most likely mode of digestion.

Predicted changes in neutron contrast are summarised in Table 2 for corn (A-type) and potato (B-type) that have undergone 20% (corn) and 7% (potato) hydrolysis. Three conditions were considered in which all the hydrolysis occurs in the (i) amorphous growth rings (background, B), (ii) amorphous lamellae (A) or (iii) crystalline lamellae (C). By introducing these modified values into the paracrystalline model, one can visualize the anticipated effects on the resultant SANS patterns; these are shown in Figure 8.

For corn, preferential hydrolysis of the crystalline lamellae gives rise to decreases in both the low q and lamellar peak intensities (compare A_{native} and $A_{\text{crystalline}}$ in Figure 8). Preferential hydrolysis of the amorphous growth ($A_{\text{amorphous}}$) rings has the effect of increasing the low q scattering intensity with no change in lamellar peak intensity. It can be readily observed that to generate both an increase in low q and lamellar peak intensities, as observed in corn (Figure 6A), hydrolysis of the amorphous lamellae ($A_{\text{amorphous}}$) must be a dominant contributor to the digestion process. This is not to say that hydrolysis does not occur elsewhere but rather that it must be operative to generate the changes observed. It is noted that while such preferential hydrolysis would, by itself, account for increases in low q scattering, additional scattering effects would inevitably arise from increases in interfacial surface area associated with increased surface pore frequency, as observed in SEM.

For potato, where only 7% digestion occurred, there were minimal changes at both low q and in the lamellar region in the kinetic SANS (Figure 6B); however, noting that the data were not normalized for the amount of starch in system - which has inevitably decreased with hydrolysis time - for a given quantity of starch, there was still an increase in the intensity in both low q and the lamellar peak albeit much less substantive than that for corn. In the case of potato, where only small changes were observed, it is hydrolysis of the amorphous growth rings that appears to be the most likely source of the scattering change (Figure 8). Erosion of the amorphous growth rings itself, combined with the opportunity for local re-organisation of the semi-crystalline lamellae would generate more efficient lateral chain packing; such behavior is consistent with observations of enhanced XRD peak resolution. It is noted, however, that the observed minor changes in potato could also be due to (i) insufficient digestion (only 7%) and (ii) the rapid initial kinetics such that the 'initial' scattering data set reflects the system in which much of the hydrolytic change has already occurred.

4. Conclusions

The action of CGTase on corn and potato starch has been investigated in terms of the kinetics of cyclodextrin production and changes in starch gelatinization behavior; in addition, the associated changes in starch granule structure arising from hydrolysis have been investigated over four orders of magnitude in spatial dimension. The action of CGTase is demonstrated to be starch type dependent and differs between corn and potato. CD production followed the trend β - > α - > γ -cyclodextrin. Although the extent of CD production was greater for corn than potato, the ratio of β -: α -: γ -CD between the two starches was conserved. In corn, surface pores facilitate action of CGTase with pores becoming more frequent with hydrolysis time but with limited changes in crystallinity and surface granule order. In contrast, the lower extent of hydrolysis and CD production results in erosion of the potato granule surface, increase in surface granule order and overall crystallinity, particularly single helical order. Hydrolysis has minimal effect on gelatinisation behaviour. Changes in the low q and lamellar peak intensities observed in the SAXS and SANS further demonstrate the different mode of action of the enzyme between the two starches. Following hydrolysis, changes in scattering in corn, are consistent with preferential attack of the amorphous lamellae, while in potato, preferential hydrolysis of amorphous growth rings is indicated. These findings provide new insight into the action of this industrially relevant enzyme. It is hoped that these insights will assist in the most appropriate selection of substrates to improve reaction yields and enable the optimisation of processing conditions for the development of novel products for enhanced encapsulation, health and nutrition.

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Figure captions

Figure 1. Extent of hydrolysis and fits thereof for corn and potato starches (A).

Cyclodextrins (α -CD, β -CD, γ -CD) released from corn (B) or potato (C) starches when subjected to hydrolysis by CGTase. Standard deviations are depicted by bars.

Figure 2. Scanning electron micrographs of corn starch and potato starch before (A, B, E and F) and after (C, D, G and H) being subjected to hydrolysis with CGTase at different magnification 1000 $\times$ (A, C, E and G) and 2500 $\times$ (B, D, F and H). Arrows highlight the abrasions observed in the granular surface.

Figure 3. Individual pore area plotted against pore frequency (related to the abundance of pores per granule) of corn (C) and potato (P) starches during CGTase hydrolysis. The symbol size is related to the hydrolysis time (0, 15, 30, 45, 60, 75, 90, 105 and 120 min).

Figure 4. (A) and (B) Details of baseline-corrected and vector normalized spectra of corn and potato starches during CGTase hydrolysis, respectively. Notations refer to the starch botanical source (Corn: C, Potato: P) followed by hydrolysis time (0, 15, 30, 45, 60, 75, 90, 105 and 120 min). (C) Ratio of absorbance intensities at 995 cm^{-1} /1022 cm^{-1} plotted as a function of hydrolysis time for corn and potato starches. Fit curves are shown as dotted lines.

Figure 5. XRD patterns for initial and final data sets for (A) corn and (B) potato starches. Notations refer to the starch botanical source (Corn: C, Potato: P) followed by hydrolysis time (0 and 120 min). Peak-fitting procedure for XRD results obtained for corn (C,D) and potato (E,F) starches at initial (C,E) and final (D,F) hydrolysis stages using Gaussian function. The area under the dashed curve indicates the amorphous content of the starch, whereas the sum of the areas under the peaks with solid lines represents the crystalline region.

Figure 6. SAXS and SANS (static and kinetic) for (A) corn and (B) potato starch.

Figure 7 – Total detector count rate versus hydrolysis time for corn and potato during kinetic SANS hydrolysis measurements.

Figure 8 – Predicted changes on SANS from hydrolysis of corn (20%) (A) and potato (7%) (B).

778 **Table 1.** Onset, peak and end point of gelatinisation transition and gelatinisation
779 enthalpy for corn and potato starch with hydrolysis time. Data are average of duplicate
780 measurements. Standard deviation on last digit in parenthesis.

Time/min	Corn				Potato			
	T _{onset} / °C	T _{peak} / °C	T _{end} / °C	ΔH _{gel} / Jg ⁻¹	T _{onset} / °C	T _{peak} / °C	T _{end} / °C	ΔH _{gel} / Jg ⁻¹
0	62.9(4)	68.9(2)	75.4(8)	10.9(9)	59.6(4)	66.5(3)	74.1(1)	15.2(3)
15	62.5(6)	69.0(6)	75.1(8)	10.5(4)	57.8(4)	62.7(4)	70(1)	13.8(4)
30	63.1(5)	69.5(4)	75.7(7)	11.2(2)	57.5(3)	62.5(2)	70.2(3)	13(2)
45	63.5(2)	69.2(8)	75.9(6)	9.7(5)	57.8(2)	62.7(3)	70.4(6)	12.8(8)
60	62.8(6)	69.2(4)	75.6(7)	11.5(3)	57.4(2)	62.2(4)	69.4(9)	13(1)
75	63.3(2)	69.7(1)	76.0(2)	10.2(8)	57.5(3)	62.6(4)	69.8(8)	12.0(7)
90	64.5(3)	70.5(3)	76.7(1)	9.57(1)	58.2(2)	63.2(1)	70.8(4)	13.2(3)
105	63.1(1)	69.2(2)	75.5(4)	11.3(1)	62.65(8)	68.87(3)	75.2(2)	13(2)
120	62.7(1)	68.9(5)	75.5(5)	8.2(7)	57.3(6)	62.3(7)	69.7(4)	12(1)

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Table 2. (i) Calculated neutron scattering length densities (SLDs) and associated contrasts for native A- and B- type granules; Change in neutron SLDs and associated contrasts for (ii) corn (A-type) starch with 20% hydrolysis and (iii) potato (B-type) starch with 7% hydrolysis. The three regions of the starch granule are denoted crystalline (C), amorphous (A) and amorphous growth ring / background (B).

Type	Granular region	Mol. density starch	Mol. density water	n SLD starch	n SLD water	Total ($10^{-6} \text{ \AA}^{-2}$)	Contrast
A-	C	1.22	0.3	1.43	-0.168	1.26	1.16
	A	0.46	0.78	0.538	-0.438	0.100	
	B	0.68	0.7	0.796	-0.393	0.403	0.302
B-	C	1.1	0.4	1.29	-0.225	1.06	0.870
	A	0.52	0.74	0.609	-0.415	0.193	
	B	0.49	0.76	0.573	-0.427	0.147	-0.0463

Region of hydrolysis (A-type)	Granular region	Mol. density starch	Mol. density water	n SLD starch	n SLD water	Total	Contrast
Amorphous	C	1.22	0.3	1.43	-0.168	1.26	1.98
	A	-0.012	1.252	-0.0140	-0.703	-0.717	
	B	0.68	0.7	0.796	-0.393	0.403	1.12
Background	C	1.22	0.3	1.43	-0.168	1.26	1.16
	A	0.46	0.78	0.538	-0.438	0.100	
	B	0.208	1.172	0.243	-0.658	-0.415	-0.515
Crystalline	C	0.748	0.772	0.876	-0.433	0.442	0.342
	A	0.46	0.78	0.538	-0.438	0.100	
	B	0.68	0.7	0.796	-0.393	0.403	0.302

Region of hydrolysis (B-type)	Granular region	Mol. density starch	Mol. density water	n SLD starch	n SLD water	Total	Contrast
Amorphous	C	1.1	0.4	1.289	-0.225	1.06	1.20
	A	0.312	0.888	0.366	-0.498	-0.133	
	B	0.49	0.76	0.573	-0.427	0.147	0.280
Background	C	1.22	0.3	1.43	-0.168	1.26	1.16
	A	0.46	0.78	0.538	-0.438	0.100	
	B	0.208	1.172	0.243	-0.658	-0.415	-0.515
Crystalline	C	0.748	0.772	0.876	-0.433	0.442	0.342
	A	0.46	0.78	0.538	-0.438	0.100	
	B	0.68	0.7	0.796	-0.393	0.403	0.302

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796 **Figure 3.**

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Figure 4.

802 **Figure 5.**

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805 **Figure 6.**

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809 **Figure 7.**

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812 **Figure 8.**

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815 **Table S1.** Position and area of peaks selected from the A-type crystal structures for the peak-fitting procedure, and crystallinity measured from the
816 XRD data according to the peak-fitting procedure for corn starch as a function of hydrolysis time.

Time (min)		0	15	30	45	60	75	90	105	120
Peak	2theta (°)	area	area	area	area	area	area	area	area	area
1	10.06	2603	1759	582	1339	570	866	627	668	2637
2	11.42	4457	3560	1912	3133	2368	2584	2302	2222	3975
3	12.59	2731	1495	1819	2658	2560	2357	2224	2609	2320
4	15.14	32802	32958	23049	28589	23331	22212	22423	21828	32890
5	17.07	40815	42792	29380	33981	29895	26143	28625	28275	40456
6	18.13	40319	40163	32429	38207	33322	28184	32533	28407	42754
7	19.92	12363	15088	13029	13660	16170	11049	17442	15288	13399
8	23.11	49220	52710	41538	43280	38551	40360	37821	33667	48067
9	24.24	14355	9958	9763	12476	11083	9893	9479	7983	16033
10	26.60	2778	3320	2186	2133	2140	2349	1967	1711	3324
11	28.87	1087	1568	664	929	738	700	16	395	1333
12	30.64	7563	7845	5698	6341	4669	2037	5439	4425	8224
13	33.52	8488	11407	6417	8142	5743	5691	6221	6370	10591
Total area		591448	541244	464219	504428	545949	489547	460923	462043	584798
Total peak area		219581	224623	168466	194868	171140	154425	167119	153848	226003
% Crystallinity (A-type polymorph)		32.10	35.78	32.14	33.74	27.91	28.16	32.18	29.32	33.11
% Crystallinity		37.13	41.50	36.29	38.63	31.35	31.54	36.26	33.30	38.65

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819 **Table S2.** Position and area of peaks selected from the B-type and V-type crystal structures for the peak-fitting procedure, and crystallinity
820 measured from the XRD data according to the peak-fitting procedure for potato starch as a function of hydrolysis time.

Time (min)		0	15	30	45	60	75	90	105	120
Peak	2theta (°)	area	area	area	area	area	area	area	area	area
1	5.76	6141	1910	1761	4001	11180	11794	11179	8282	15163
2	10.37	5571	483	2265	2859	4075	2814	4128	793	4309
3	11.51	0	0	0	0	2714	3527	2697	187	2791
4	14.17	31411	24003	26658	25426	11226	7828	11815	3188	4679
5	15.13	0	0	0	0	14278	25903	13503	7014	33150
6	17.20	57241	51979	47853	48292	47073	56126	47204	23695	63236
7	19.68	4351	3004	3676	3652	4193	3740	4281	787	5062
8	22.40	22955	27525	21880	23170	18910	22145	19010	12127	25111
9	24.08	18407	5058	13242	13859	17323	18718	17419	12237	22501
10	26.46	1769	3053	617	1145	1973	2254	2080	1123	3559
11	30.52	4581	1903	2425	2766	6378	5413	5344	1142	6852
12	31.45	0	0	0	0	0	314	538	1060	1014
13	34.20	4753	2065	2500	4179	5916	4134	6081	1654	4195
Total area		558168	479763	517777	502741	479772	532778	380695	196506	481583
Total peak area		157180	120983	122876	129348	145239	164710	145279	73289	191622
% Crystallinity (B-type polymorph)		24.07	23.26	21.88	22.76	23.55	25.24	29.74	30.05	23.02
% Crystallinity (V-type polymorph)		4.09	1.96	1.85	2.97	6.72	5.68	8.42	7.25	16.77
% Crystallinity		28.16	25.22	23.73	25.73	30.27	30.92	38.16	37.30	39.79

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823 **Figure S1.** Evolution of the XRD patterns as a function of hydrolysis time for (A) corn and (B) potato starches. Notations refer to the starch
824 botanical source (Corn: C, Potato: P) followed by hydrolysis time (0, 15, 30, 45, 60, 75, 90, 105 and 120 min).

827 **Figure S3** – Static SAXS and SANS from Potato control samples.

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