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Analysis of a preferential action of α-amylase from *B. licheniformis* towards amorphous regions of waxy maize starch

María Laura Foresti[a,b,*], María del Pilar Williams[a], Ricardo Martínez-García[a,b], Analía Vázquez[a,b]

[a] Instituto de Tecnología en Polímeros y Nanotecnología (ITPN), Facultad de Ingeniería, Universidad de Buenos Aires, Capital Federal, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

Waxy maize starch was subjected to α-amylase (*Bacillus licheniformis*) hydrolysis in buffered medium to determine the evolution of reaction in quantitative terms and also in terms of the morphology and crystallinity of the partially hydrolyzed starch granules. Gathered data allowed studying the pattern of action of this α-amylase over waxy maize starch granules, with particular focus on a preferential hydrolysis of the amorphous regions of starch. Results showed that waxy maize starch hydrolysis followed a two-stage kinetic profile with an initial stage characterized by high reaction rate, followed by a slower second stage. The change of hydrolysis rate occurred at approximately 6 h of reaction, a time for which X-ray diffraction data quantitatively analyzed by three different techniques showed a maximum of crystallinity in partially hydrolyzed granules. Scanning electron microscopy images illustrated the action of α-amylases which implied the exoerosion of the granules surface, the entry of α-amylases into the granules through radial channels, their endoerosion towards the granule exterior, and their fragmentation. Fragmentation of waxy maize starch granules revealed internal layered structures of starch which were interpreted as hydrolyzed/non-hydrolyzed growth rings. Under the conditions chosen, kinetic, electron microscopy and X-ray data all gave evidence of a preferential action of α-amylase from *Bacillus licheniformis* towards the less ordered regions of waxy maize starch. Results showed that, provided the proper hydrolysis time is chosen, starch granules with increased crystallinity can be obtained by a pure enzymatic treatment.

1. Introduction

Starch is a natural, renewable, and biodegradable polymer produced by many plants as a source of stored energy (Le Corre, Bras, & Dufresne, 2010). Starch is found in plant roots, stalks, crop seeds, and staple crops such as rice, corn, wheat, tapioca, and potato (Buléon, Colonna, Planchot, & Ball, 1998). In human nutrition, starch plays a major part in supplying the metabolic energy that enables the body to perform its different functions (Miao, Zhang, Mu, & Jiang, 2011). Although starch structure has been under research for years, its complexity has promoted that a universally accepted model is not still available (Le Corre et al., 2010). However, a multiscale structure of the granule into which alternating amorphous and semicrystalline growth rings (120–500 nm) composed of blocklets (20–50 nm) made of amorphous and crystalline lamellae (9 nm) containing amylopectin and amylose chains (0.1–1 nm) are found, has been reported to be the most widely accepted model of the last years (Le Corre et al., 2010). The characteristic layered structure of starch granules due to the so-called growth rings often seen by optical, scanning and transmission electron microscopy is the result of multiple concentric shells of increasing diameter extending from the hilum towards the surface of granules generated by (periodical) diurnal deposition of starch (Tester, Karkalas, & Qi, 2004).

Enzymatic and acid hydrolyses have been used traditionally to modify native starches and to create products with altered solubility, viscosity, and/or gelation properties that find broad applications in food, paper, textile, and other industries (Hoover, 2000; Wang & Wang, 2001). α-Amylase is an endo-specific enzyme that randomly catalyzes the hydrolysis of α-(1→4) glycosidic linkages in amylose and amylopectin chains. In the last years, α-amylase-catalyzed hydrolysis of starch has received much attention due to its industrial value for the production of glucose and fructose syrups and other starch hydrolysates.

In this contribution, the focus of analysis is the partially hydrolyzed starch granules resulting from α-amylase-catalyzed hydrolysis under definite conditions. In this context, kinetic, morphological, and X-ray diffraction data, are used in combination

* Corresponding author at: Instituto de Tecnología en Polímeros y Nanotecnología (ITPN), Facultad de Ingeniería, Universidad de Buenos Aires, Capital Federal, Argentina. Tel.: +54 11 45143010; fax: +54 11 45143010.
E-mail address: mforesti@fi.uba.ar (M.L. Foresti).

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to study the action of α-amylase from *B. licheniformis* during the progress of waxy maize starch hydrolysis. A review of specific literature of the last years shows that a number of authors agree on the fact that during α-amylolysis of starch, the less ordered of the granule are more easily and rapidly hydrolyzed than the more ordered crystalline layers (Gallant, Bouchet, Buléon, & Perez, 1992; Gallant, Bouchet, & Baldwin, 1997; Kim, Park, & Lim, 2008; Oates, 1997; Tester et al., 2004; Wang & Wang, 2004; You & Izydorczyk, 2007). However, and differently for example from endoglucanases which are known to be cellulases specific of the amorphous regions of cellulose, α-amylases are not specific of the amorphous regions of starch, and can also exert their hydrolysis action over more ordered regions. In this context, there are authors who have given experimental evidence that amylolysis proceeds simultaneously in amorphous and ordered regions of the starch granules. This is the case of Miao et al. (2011) who studied the hydrolysis of waxy maize starch by use of a porcine pancreas α-amylase and an amyloglucosidase. Upon hydrolysis authors found that the measured enthalpy of gelatinization, crystal structure, and crystallinity of starch granules remained unvaried, which authors interpreted as evidences of simultaneous enzymatic hydrolysis of both crystalline and amorphous regions of starch. Recently, Le Corre, Vahanian, Dufresne, and Bras (2012) developed a 2-h enzymatic pretreatment for a faster production of starch nanocrystals by acid hydrolysis. From kinetic data, atomic force microscopy and X-ray diffraction results authors reported that the enzymatic pretreatment with β-amylase or glucoamylase created porosity by hydrolyzing *indifferently* amorphous and crystalline starch. In the work of Lauro, Forssell, Suortti, Hulleman, and Poutanen (1999) authors concluded based on X-ray diffraction data that both amorphous and semi-crystalline regions of barley starch granules were hydrolyzed simultaneously by α-amylase at the initial stage of hydrolysis.

In this context, the main aim of this work was to study the evolution of the hydrolysis of waxy maize starch catalyzed by α-amylase from *Bacillus licheniformis*, and determine whether under the chosen conditions hydrolysis proceeds preferentially in the amorphous regions of starch. Moreover, in a case of a preferential action towards the less ordered regions of starch, another aim of this contribution was to determine the conditions required to isolate partially hydrolyzed starch granules with increased crystallinity.

2. Experimental

2.1. Materials

Waxy maize starch (97%) was kindly donated by Ferromet (Argentina) which commercializes waxy maize starch provided by Roquette (France). α-Amylase from *Bacillus licheniformis* with a molecular weight of 62 kDa was purchased from Sigma–Aldrich as a saline sucrose solution containing 21.0 mg/mL protein (Biuret), and 623 units/mg protein. D-(+)-Maltose monohydrate standard and the reagents used in the colorimetric determination of reducing sugars (potassium sodium tartrate tetrahydrate and 3,5-dinitrosalicylic acid (DNS)), were all bought from Sigma–Aldrich. Buffer pH 7 was bought from Laboratorios Olivieri (Argentina). All other reagents used were of analytical grade.

2.2. Starch hydrolysis

1 g of starch was dispersed in 30 mL of buffer of pH 7 kept at 40 °C and 350 rpm. 785 U of α-amylase were added and this was defined as the beginning of reaction. Suspensions were kept at constant temperature and magnetic stirring during 24 h. Samples (300 μL) were withdrawn at definite reaction time intervals (1, 2, 4, 6, 8, 24 h), filtered through a 0.22 μm membrane, and properly diluted in order to inspect the hydrolysis kinetics by the DNS method using maltose as standard. After the chosen reaction time, the solid product was separated by vacuum filtration in a Buchner funnel. Several washings of the solid recovered with distilled water were performed in order to guarantee the removal of the biocatalyst and soluble hydrolysis products. The solid was finally dried overnight in an air oven at 40 °C. The evolution of reaction was also determined by a parallel set of reactions, each of which was stopped at selected hydrolysis times to recover the remaining insoluble starch residue. The extent of starch hydrolysis (%) achieved at each reaction interval was calculated as the amount (g) of maltose equivalents released per gram of dried starch before treatment × 100 (double beam PG Instruments T80 UV/VIS Spectrophotometer) using the DNS method (Miller, 1959); as well as by the fraction of solubilized starch (i.e. (1 − weight of the dried remaining insoluble solids)/weight of dried starch before treatment × 100).

2.3. Characterization of partially hydrolyzed granules

Drops of partially hydrolyzed starches/water suspensions (1%, w/v) were deposited on microscope glasses and dried at 40 °C for 5 min. Samples were then coated with gold using an ion sputter coater, and observed by use of a scanning electron microscope Zeiss Supra 40 with field emission gun operated at 3 kV. At each hydrolysis time chosen, the granules size distribution as well as the growth rings thickness distribution were determined by use of ImageJ and JMicroVision v.1. 2.7. software according to a length scale provided by the user. More than 35–60 granules/growth rings were considered for each determination.

X-ray diffraction (XRD) analysis was performed on native and selected hydrolyzed starches in a Rigaku diffractometer with Bragg Bentano geometry, and Cuα radiation in the range of $2\theta = 5–50°$. Samples were measured under temperature and humidity room conditions. The diffractograms obtained were carefully smoothed and normalized with respect to the highest intensity. The crystallinity of native and partially hydrolyzed starch granules was then quantified in terms of the crystallinity index (CI), a parameter frequently used to describe the relative amount of crystalline material in a sample. Three different methods dispersedly found in the literature for the determination of crystalline and amorphous contributions in starch were used, namely the "two-phase" method adapted from Nara and Komiya (1983); a deconvolution method assuming Lorentzian peaks which fitted experimental XRD data by use of Origin software, and an adaptation of Segal' method derived and widely used for a rapid estimation of cellulose crystallinity index (Segal, Creely, Martin, & Conrad, 1962).

3. Results and discussion

3.1. Kinetics of starch hydrolysis

The techniques most widely used to determine the extent of enzymatic hydrolysis of starch are based in UV/visible spectroscopy determinations of breakdown products (e.g. Bruner, 1964; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Miller, 1959) and weight loss measurements (e.g. Le Corre et al., 2012; You & Izydorczyk, 2007). Fig. 1 illustrates the evolution of waxy maize starch hydrolysis catalyzed by *B. licheniformis* in terms of mass of maltose produced, remaining insoluble starch mass, and extent of starch hydrolysis (%) calculated by both methods described.

The pattern of waxy maize starch hydrolysis evolution determined by both methods used shows two distinct stages of hydrolysis with different reaction rate. The first higher rate stage

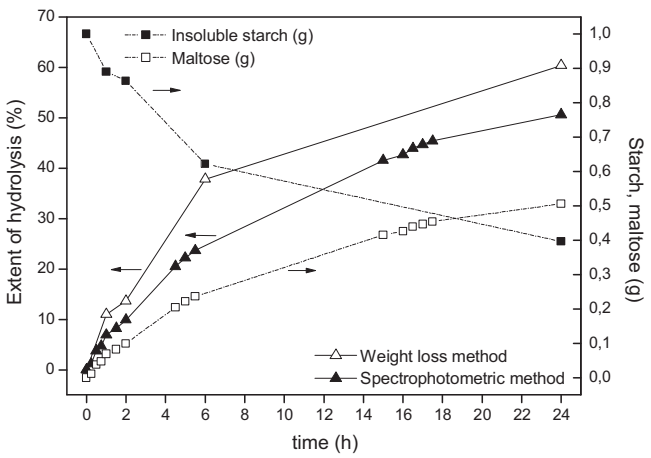

Fig. 1. Evolution of waxy maize starch hydrolysis catalyzed by α-amylase from *B. licheniformis*, 785 U, 40 °C. Extent of hydrolysis (%) measured by weight loss and spectrophotometric methods. Evolution of the mass of remaining insoluble starch and produced maltose (g).

is observed up to approximately 6 h of reaction. In the following period assayed (6–24 h) a slower evolution of starch hydrolysis is observed. Similar two-stage patterns have been reported by other authors (Gallant et al., 1992; Kim et al., 2008; Planchot, Colonna, Gallant, & Bouchet, 1995), who have explained their results by assuming that they are the consequence of hydrolysis occurring mainly in the amorphous region during the first stage, followed by a later hydrolysis of the crystalline regions at a lower rate. In the early contribution of Planchot et al. (1995) dealing with the hydrolysis of starch granules of various botanical origins catalyzed by an α-amylase from *A. fumigatus*, two-stage erosion was evident for all starches except potato starch. The first stage was characterized by high hydrolysis rates which, together with the corresponding hydrolysis time periods, varied according to starch type (Planchot et al., 1995). Besides the structural features of the starch substrate (i.e. zones with different crystallinity) the observed variations in the rate of hydrolysis in the two stages described, have less seldom been attributed to the appearance of oligosaccharides that may act as competitive inhibitors in solution (Leloup, Colonna, & Ring, 1992; Planchot et al., 1995). The hypothesis of an initial fast hydrolysis of amorphous followed by a slower hydrolysis of crystalline regions has also been used to explain the two-stage hydrolysis profiles frequently evidenced during acid-catalyzed hydrolysis of starch granules (Biliaderis, Grant, & Vose, 1981; Dufresne & Cavaillé, 1998; Jayakody & Hoover, 2002). Even three steps of acid-catalyzed hydrolysis have been distinguished by some authors, who have attributed them to the hydrolysis of amorphous layers, semicrystalline layers and crystalline layers of starch, respectively (Angellier, 2005; Li, Corke, & Beta, 2007).

Despite their very similar two-stage pattern, comparison of quantitative results obtained herein by use of gravimetric and spectrophotometric methodologies reveal slight differences in hydrolysis extent values observed. The differences, with values that are always higher for the hydrolysis extent derived from the weight loss measurement, may be attributed to soluble intermediate oligosaccharide products which are quantified by weight loss determinations but which cannot be detected by the spectrophotometric method/have a lower response than maltose.

3.2. Evolution of granules morphology (scanning electron microscopy, SEM)

Fig. 2 illustrates the action of α-amylase from *B. licheniformis* over waxy maize starch granules for increasing hydrolysis times. SEM micrographies chosen are representative of the predominant state of starch granules at the target times. Fig. 2(a–b) shows native waxy maize starch granules which still have not been exposed to α−amylase action. Waxy maize starch granules show a polyhedral shape with smooth surfaces. The analysis of the size distribution evidenced granules diameters in the range of 3–21 μm, with an average diameter of 11 μm. Numerous pinholes were observed on some of the waxy maize starch granules (Fig. 2(b)), which have been proposed to be openings to channels/pores which provide access to hydrolysing enzymes to the granule interior (Huber & BeMiller, 2000). Oates (1997) reported that the entry of hydrolysing enzymes and other large molecules into the interior of starch granules is restricted and only possible through pores or channels, which are either naturally occurring features of the granule, or the consequences of damages that may have taken place during granules extraction. The evolution of starch granules morphology during hydrolysis (Fig. 2(c–h)) may be explained by the current knowledge of the action of α-amylases over starch granules. Enzymatic reaction with insoluble substrates such as starch granules occurs via three steps: diffusion to the solid surface, adsorption, and finally catalysis. Once that the enzyme is adsorbed onto the granules surface, starch hydrolysis initiates as a superficial erosion phenomenon as illustrated in Fig. 2 (c). Following the initial attack, hydrolytic enzymes find access to the interior of the granules via channels, leaving holes that enlarge with hydrolysis time, as illustrated in Fig. 2(d–e). Subsequent enzyme attack proceeds from the interior to the outwards of the granules.

Micrographies corresponding to 6 h of hydrolysis (i.e. Fig. 2(f)) evidence the presence of some fragmented granules in whose interior hydrolyzed/non-hydrolyzed layers, which resemble the growth rings of starch, can be identified. In the hydrolysis of normal and waxy maize starches catalyzed by α-amylase from *A. fumigatus*, Planchot et al. (1995) also observed highly eroded layered structures by use of scanning or transmission electron microscopy. Authors concluded that the emergence of a layered internal structure in the partially hydrolyzed granules suggested the existence of superimposed layers of high and low susceptibilities related to different crystallinity levels. Under the hypothesis that α-amylases hydrolyze amorphous regions at a higher rate than more ordered regions, these hydrolyzed/non-hydrolyzed may be assigned to amorphous-hydrolyzed/semicrystalline-less-hydrolyzed growth rings, respectively.

In reference to granules fragmentation, previous works have revealed that at sufficiently long amylolysis periods some granules became split open revealing their internal layered structure (Li, Vasanthan, & Bressler, 2012; Miao et al., 2011). Kim et al. (2008), hypothesized that the erosion of the amorphous regions of the granules induced their fragmentation. With the increase of hydrolysis time, the fraction of fragmented particles herein observed increased, exposing the interior of a higher fraction of granules with the described hydrolyzed/non-hydrolyzed layered pattern (Fig. 2(g)). Granules fragmentation was also evidenced by the progressive reduction of the average granules diameter. Size distributions found in this contribution for increasing hydrolysis time showed average diameters that reduced from 11 μm to 7.7 μm in 24 h of hydrolysis. At reaction times higher than 48 h, SEM images suggested partial gelatinization of starch which is illustrated by partially hydrolyzed starch granules which appeared “covered” by an amorphous gel (Fig. 2(h)). Partial gelatinization could have been caused by extensive shear during long periods with magnetic stirring.

Magnified images of the interior of 24 h-hydrolyzed-granules showing layered patterns of alternating hydrolyzed/non-hydrolyzed growth rings have been included in Fig. 3. Measurement of the thickness of each zone by use of JMicroVision software allowed obtaining an average thickness of each zone. Considering

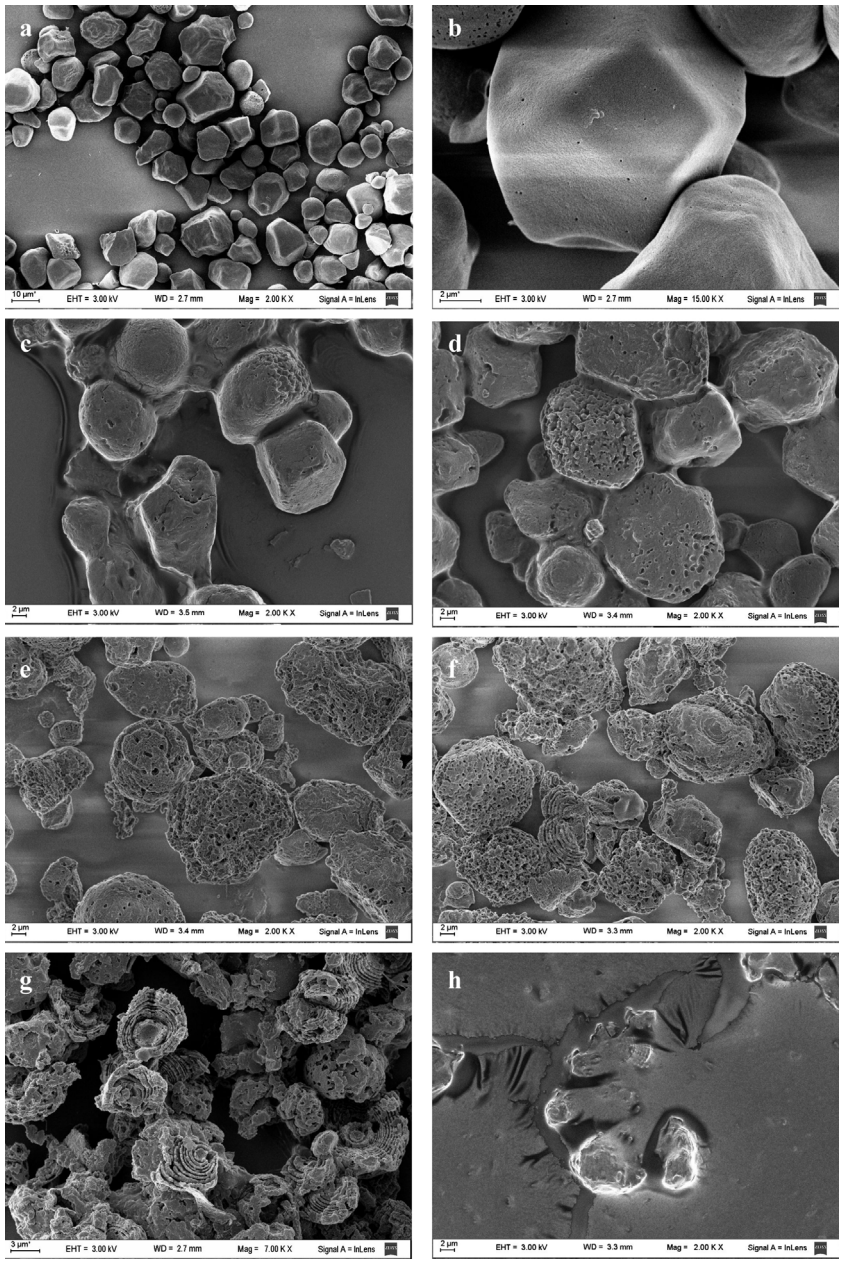

Fig. 2. Action of α-amylase over granules morphology during hydrolysis. (a and b) Native waxy maize starch – 0 h, (c) 1 h, (d) 2 h, (e) 4 h, (f) 6 h, (g) 24 h, (h) 72 h.

34 measures (see measure indications in magnified images of fragmented granules included in Fig. 3(b, d and f)), the amorphous hydrolyzed growth rings showed an average thickness of 107 nm with a standard deviation of 44 nm. In the case of semi-crystalline non-hydrolyzed growth rings, the average thickness found upon 56 measures was 137 nm with a standard deviation of 40 nm. Average values obtained are in agreement with the thicknesses of alternating amorphous and semicrystalline growth rings reported in the literature (Le Corre et al., 2010; Tester et al., 2004). According to the model of Cameron and Donald (1992) starch granules contain relatively broad radial growth rings comprising semi-crystalline shells which are about 140 nm thick, separated by broad amorphous zones of at least the same thickness (Tester et al., 2004). In the review of Le Corre et al. (2010) authors summarize the current knowledge of starch multiscale structure in which growth rings have thicknesses in the range of 120–500 nm. Average thicknesses determined for hydrolyzed/non-hydrolyzed regions contribute to the hypothesis of a preferential action of α-amylases towards amorphous regions of starch granules which appear as void hydrolyzed concentric rings. Fig. 3(g–h) illustrates the layered interior of unfragmented 24 h-hydrolyzed-waxy maize starch granules.

3.3. Evolution of granules crystallinity (X-ray diffraction analysis)

Starch is semicrystalline in nature with varying levels of crystallinity (i.e. 15–45% according to Zobel (1988), 15–51% according to Tester et al. (2004)). In normal and waxy starches, the branches of amylopectin form double helices that are arranged in crystalline domains. On the other hand, amorphous regions consist mainly of linear amylose chains and amylopectin branching points. According to the packing of the amylopectin double helices different polymorphic forms of starch can be found, which account for the A, B, and C X-ray patterns. Native waxy maize starch used in this

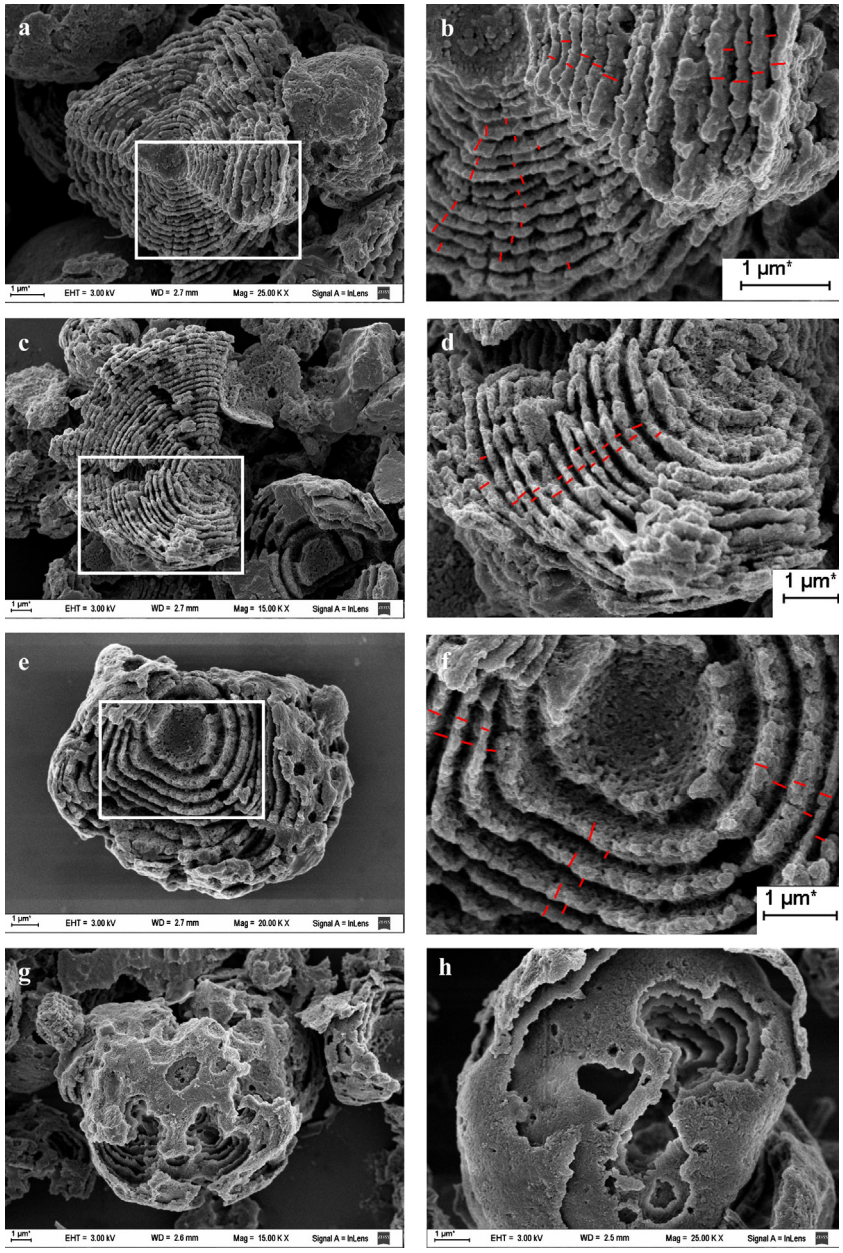

Fig. 3. Micrographies of the interior of partially hydrolyzed waxy maize starch granules (hydrolysis time: 24 h). (b, d and f) Magnified images of selected regions.

contribution exhibited a typical A-type X-ray diffraction pattern, with the peaks at Bragg angles (2θ) of 14.9, 16.9, 17.7, and 22.8° as previously reported for waxy maize starch (e.g. Angellier, Molina-Boisseau, Lebrun, & Dufresne, 2005; Le Corre et al., 2012).

Given the difficulties in isolating the crystalline and amorphous contributions in XRD data of starch, quantitative analysis is not very frequently reported. In the current contribution, quantitative determination of native and partially hydrolyzed samples crystallinity in terms of their crystallinity index (CI, defined as the ratio of the amount of crystalline starch to the total amount of sample material) was performed by use of three different methods found in the literature. As recently described by Le Corre et al. (2012), when analyzing X-ray diffractograms of starch, several methods to calculate starch crystallinity can be considered. One method frequently used is the so-called "*two-phase method*", in which the area above a curve connecting the peak baselines is assumed to correspond to the area of crystalline domains, whereas the lower area is assumed to correspond to the amorphous part (Nara & Komiya, 1983). CI results obtained herein by the mentioned method were calculated as illustrated in Fig. 4(a). The interval considered was $2\theta = 5–27°$, since it is where the *main* crystalline peaks are found. The explicit inclusion of the zone of the diffractogram of higher 2θ values in the calculus of CI (i.e. $2\theta = 27–50°$) of course modifies the absolute values obtained, but it must be pointed out that the pattern of CI versus hydrolysis time found (including the 6 h maximum shown later on in Fig. 5), is exactly the same (data not shown). Values of CI obtained by consideration of the whole diffractogram (i.e. $2\theta = 5–50°$) are lower than the ones obtained according to Fig. 4(a) due to the fact that crystalline peaks found at high 2θ angles contribute only slightly to the crystalline fraction.

A more accurate, although more time-consuming, method for crystallinity index determination is the *deconvolution method*, in which by use of specific software amorphous and crystalline contributions to the diffraction spectrum are separated using a curve-fitting process. Curve-fitting requires that the shape and number of peaks are assumed. In the current contribution

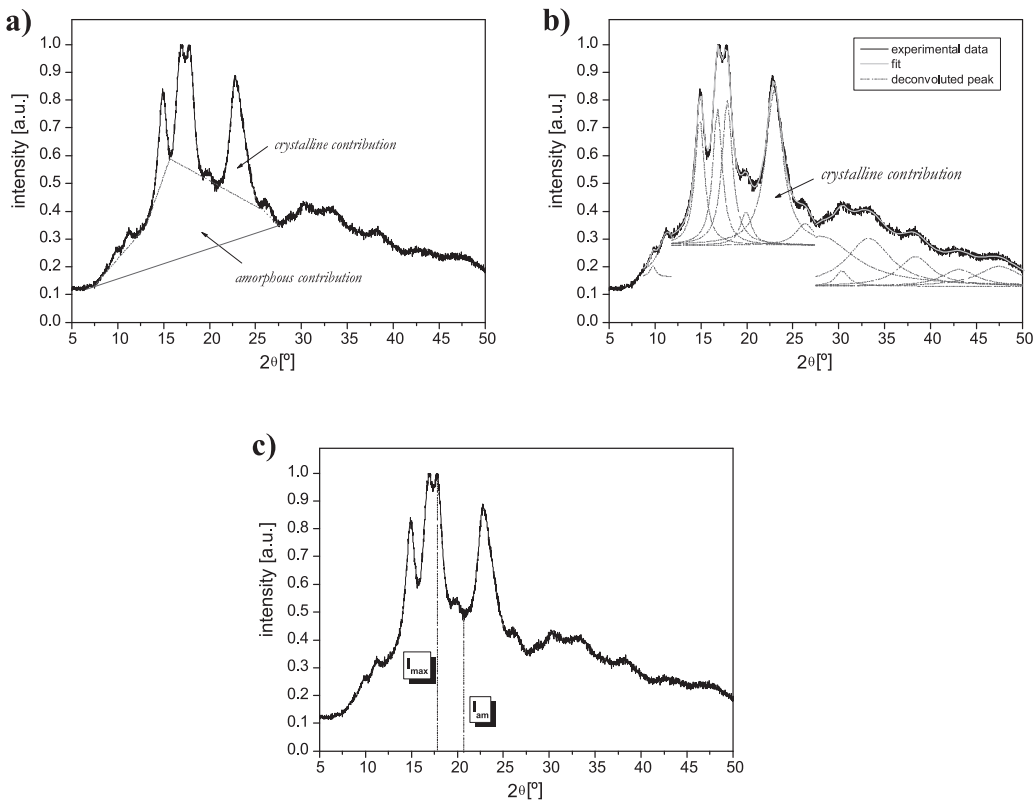

Fig. 4. Scheme of CI calculation methods. (a) Two-phase method, (b) deconvolution method, (c) Segal's method.

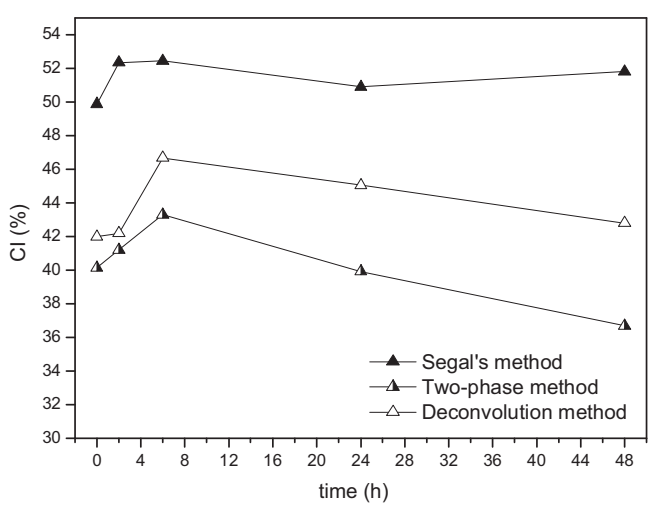

Fig. 5. Evolution of the crystallinity index (%) of partially hydrolyzed waxy maize starch granules, determined by three methods found in the literature.

Lorentzian functions have been chosen for XRD spectra deconvolution. CI is calculated from the ratio of the area of all deconvoluted crystalline peaks to the total diffractogram area (Fig. 4(b)).

Finally, as an adaptation of the *peak height method* developed by Segal et al. (1962) for calculating cellulose's crystallinity index, the recent contribution of Le Corre et al. (2012) proposed the calculation of CI of starch from the height ratio between the intensity of the highest crystalline peak ($I_{max} - I_{am}$) and total intensity (I_{max}) (Fig. 4(c)). For calculations performed in this contribution I_{max} corresponds to the total intensity of the diffractogram measured at $2\theta = 17.7^\circ$, and I_{am} corresponds to the intensity of the diffractogram at the minimum between the crystalline peaks found at $2\theta = 17.7^\circ$ and 22.8° (i.e. $2\theta = 21^\circ$), which is assumed to be representative of the amorphous contribution. A scheme of the methods used for CI determination is given in Fig. 4 for clarity purposes. The results of the evolution of waxy maize starch granules′CI with hydrolysis time by all three methods described have been summarized in Fig. 5.

Fig. 5 illustrates the evolution of CI values during α-amylase hydrolysis (time interval 0–48 h) calculated by the three methods described. Values of CI are all in the 37–52% interval. The fact that values of CI for a definite hydrolysis time vary depending on the choice of measurement method used is a feature in common with cellulose CI determination (Evans, Newman, & Roick, 1995; He, Cui, & Wang, 2008; Park, Baker, Himmel, Parilla, & Johnson, 2010; Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005). As shown in Fig. 5, and similarly to the behaviour observed in the comparison of cellulose CI calculated by the methods described (Park et al., 2010), the adaptation of Segal's method provides the highest CI values for native and partially hydrolyzed waxy maize starch granules. Variations in CI determined by this technique are low, although a slight increase in the CI promoted by enzymatic hydrolysis during the initial hours of amylolysis is observed. As in the case of the use of this method for the determination of the CI of cellulose samples, Segal' method deficiencies arise from the use in the calculation of just one crystalline peak out of *at least* four, and from the fact that the variation in peaks width is neglected (Park et al., 2010). In this context, the height method should be considered as a rough approximation of the CI value for a rapid comparison between samples. On the other hand, and as in the case of cellulose, the other XRD methods proposed herein which consider the contributions from both amorphous and crystalline cellulose to the whole of the XRD spectrum, provide a more accurate measure of the crystallinity of starch samples.

Although the two-phase and the deconvolution methods do not give exactly the same CI values for a given starch sample, the pattern of CI evolution (increase with respect to native starch CI

value in the 2–6 h interval followed by reduction of CI values in the 6–48 h interval) is very similar for both methods. The described increase-decrease CI pattern may be attributed to the discussed preferential action of α-amylases over the amorphous zones of starch. A faster enzymatic removal of the less ordered regions of starch should result in an *initial* increase in the samples CI values, as the ones shown in Fig. 5. By the way, all three methods used for CI determination exhibit highest values at around 6 hours of hydrolysis, which interestingly coincide with the time interval for which a reduction of the hydrolysis rate was observed by both quantitative methods used to follow hydrolysis kinetics (Fig. 1). An increase in starch crystallinity upon amylolysis has previously reported for other systems. In the contribution of Zhou, Hoover, and Liu (2004) the X-ray patterns of the hydrolyzed residues of wrinkled pea starch showed that the relative crystallinity increased substantially on hydrolysis (from 17.8 to 33.4%). Authors attributed these results to the extensive degradation of the amorphous regions of the starch granule. Based on differential scanning calorimetric results, You and Izydorczyk (2007) also concluded that the overall crystalline order in zero amylose starch increased due to partial α-amylolysis. On the other hand, and based on the observation that α-amylolysis did not produce an increase in crystallinity, a number of researchers have concluded that α-amylases can simultaneously solubilize both amorphous and crystalline regions of starch granules (Colonna, Buléon, & Lemarié, 1988; Lauro et al., 1999; Le Corre et al., 2012; Leach & Schoch, 1961; Zhou et al., 2004).

Opposite results found by different authors could be explained with concepts similar to those proposed by Park et al. (2010) for the variations of cellulose CI upon enzymatic hydrolysis. As previously briefly reviewed for enzymatically hydrolyzed starch, the reported changes in CI after enzymatic hydrolysis of cellulose do not show a clear trend either (Park et al., 2010). Even if there are many studies supporting the idea that CI increases during enzymatic hydrolysis of cellulose, the reported increases have often been small (i.e. ≈2–2.6%). Moreover, a number of other reports did not find any change in CI values after enzymatic action (Park et al., 2010). As stated in the mentioned work, these results make it unclear whether there is a preferential digestion of the amorphous cellulose component, even if highly amorphous celluloses have shown to be much more susceptible to cellulase action than native cellulose (Zhang, Cui, Lynd, & Kuang, 2006). Similarly, although gelatinized amorphous starch is known to be enzymatically hydrolyzed at a much higher rate than semi-crystalline granular starch, many articles referenced herein found no differences in CI values upon amylolysis; whereas other positive CI changes observed (as the ones determined in this contribution, i.e. 3–5%) have usually been low. The previous might be explained by the hypothesis of enzymes acting initially on amorphous regions of highest accessibility, but concomitally promoting some grade of disorder of starch regions with higher crystallinity. As proposed in the work of Park et al. (2010) even if enzymes work initially on cellulose microfibril surfaces consuming the less ordered surface layers of cellulose, then internal ordered cellulose chains become surface chains with decreased order, so that conversion of 'amorphous cellulose' results in production of more 'amorphous cellulose' with a further decrease in cellulose CI. In this context, a possible explanation for the low increases observed herein in CI during enzymatic hydrolysis (whereas kinetic and SEM data suggest a more notorious preference of α-amylase towards amorphous regions of starch); could be attributed to the hypothesis that while amylolysis proceeds preferentially and at a higher rate on the less ordered regions of starch granules, semi-crystalline fractions of starch simultaneously decrease their order. The previous hypothesis of a concomitant action of the phenomena described (one that induces crystallinity increment and the other that reduces it), may justify the small crystallinity changes often observed by most authors during α-amylase hydrolysis of starch; whereas there are other studies in which no significant change in starch crystallinity was observed (and thus it was concluded that – at least during the assay interval studied – hydrolysis occurred simultaneously in both order and disordered regions of starch). Moreover, simultaneous partial disorganization of starch induced by shear promoted during long hydrolysis intervals with enough stirring speed required to keep starch in suspension, may also be a plausible cause of crystallinity reduction.

Finally, and going back to the increasing-decreasing pattern of CI illustrated in Fig. 5, reduction of CI after the 6 h period clearly observed by use of the deconvolution and two-phase methodologies, suggests that extensive hydrolysis effectively destroys and solubilizes the crystalline areas of the granules. Lauro et al. (1999) also observed that crystallinity and gelatinization enthalpy of barley starches decreased during the later stages of α-amylolysis, which Zhou et al. (2004) attributed to extensive hydrolysis that effectively destroyed and solubilized the crystalline areas of starch granules.

4. Conclusion

Waxy maize starch amylolysis catalyzed by α-amylase from *Bacillus licheniformis* was studied in terms of the quantitative evolution of the hydrolysis reaction, as well as in terms of morphological and crystallinity characteristics of partially hydrolyzed starch granules. The two methods used to determine reaction kinetics showed a change of reaction rate at approximately 6 h of reaction, delimiting two hydrolysis stages which have previously been attributed to the hydrolysis of starch regions with different crystallinity. Besides, for the mentioned hydrolysis time, the use of two different methods for CI calculation showed a maximum of crystallinity in residual starch granules. Scanning microscopy images illustrated the pattern of α-amylase action over starch granules, with the most interesting feature being the generation of clear layered hydrolyzed/non-hydrolyzed interior structures, which suggested different susceptibility to enzyme attack of alternating growth rings. Image analysis allowed finding very good estimations of the growth rings thicknesses which agreed with values found in the literature.

All three type of analysis performed herein (kinetic data, SEM and XRD analysis), contributed to the validation of the hypothesis that under the assay conditions chosen, in the initial hours of reaction amylolysis effectively proceeds preferentially and at a higher rate in the less ordered regions of starch. Moreover, results showed that if reaction is stopped at 6 h of hydrolysis, micrometric starch granules with increased crystallinity obtained by a pure enzymatic protocol can be easily produced.

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References

Angellier, H. (2005). *Nanorenforts d'amidon de maïs cireux pour applications nanocomposites*. Grenoble, France: University Joseph Fourier (Ph. D. Thesis).

Angellier, H., Molina-Boisseau, S., Lebrun, L., & Dufresne, A. (2005). Processing and structural properties of waxy maize starch nanocrystals reinforced natural rubber. *Macromolecules*, *38*, 3783–3792.

Biliaderis, C. G., Grant, D. R., & Vose, J. R. (1981). Structural characterization of legume starches. I. Studies on amylose, amylopectin and beta-limit dextrins. *Cereal Chemistry*, *58*, 496–502.

Bruner, R. L. (1964). Determination of reducing value: 3,5-Dinitrosalicylic acid method. In R. L. Whistle (Ed.), *Methods in carbohydrate chemistry* (pp. 67–71). New York/London: Academic Press.
Buléon, A., Colonna, P., Planchot, V., & Ball, S. (1998). Starch granules: Structure and biosynthesis. *International Journal of Biological Macromolecules*, *23*, 85–112.
Cameron, R. E., & Donald, A. M. (1992). A small angle X-ray scattering study of the annealing and gelatinization of starch. *Polymer*, *33*, 2628–2635.
Colonna, P., Buléon, A., & Lemarié, F. (1988). Action of *Bacillus subtilis* α-amylase on native wheat starch. *Biotechnology and Bioengineering*, *31*, 895–904.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substance. *Analytical Chemistry*, *28*, 350–356.
Dufresne, A., & Cavaillé, J.-Y. (1998). Clustering and percolation effects in microcrystalline starch-reinforced thermoplastic. *Journal of Polymer Science: Part B: Polymer Physics*, *36*, 2211–2224.
Evans, R., Newman, R. H., & Roick, U. C. (1995). Changes in cellulose crystallinity during kraft pulping. Comparison of infrared, X-ray diffraction and solid state NMR results. *Holzforschung*, *49*, 498–504.
Gallant, D. J., Bouchet, B., & Baldwin, P. M. (1997). Microscopy of starch: Evidence of a new level of granule organization. *Carbohydrate Polymers*, *32*, 177–191.
Gallant, D. J., Bouchet, B., Buléon, A., & Perez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. *European Journal of Clinical Nutrition*, *46*, 3–16.
He, J., Cui, S., & Wang, S.-Y. (2008). Preparation and crystalline analysis of high-grade bamboo dissolving pulp for cellulose acetate. *Journal of Applied Polymer Science*, *107*, 1029–1038.
Hoover, R. (2000). Acid-treated starches. *Food Review International*, *16*, 369–392.
Huber, K. C., & BeMiller, J. N. (2000). Channels of maize and sorghum starch granules. *Carbohydrate Polymers*, *41*, 269–276.
Jayakody, L., & Hoover, R. (2002). The effect of lintnerization on cereal starch granules. *Food Research International*, *35*, 665–680.
Kim, J.-Y., Park, D.-J., & Lim, S.-T. (2008). Fragmentation of waxy rice starch granules by enzymatic hydrolysis. *Cereal Chemistry*, *85*, 182–187.
Lauro, M., Forssell, P. M., Suortti, M. T., Hulleman, S. H. D., & Poutanen, K. S. (1999). α-Amylolysis of large barley starch granules. *Cereal Chemistry*, *76*, 925–930.
Le Corre, D., Bras, J., & Dufresne, A. (2010). Starch nanoparticles: A review. *Biomacromolecules*, *11*, 1139–1153.
Le Corre, D., Vahanian, E., Dufresne, A., & Bras, J. (2012). Enzymatic pretreatment for preparing starch nanocrystals. *Biomacromolecules*, *13*, 132–137.
Leach, H. W., & Schoch, T. J. (1961). Structure of the starch granule II: Action of various amylases on granular starches. *Cereal Chemistry*, *76*, 925–930.
Leloup, V. M., Colonna, P., & Ring, S. G. (1992). Physicochemical aspect of resistant starch. *Journal of Cereal Science*, *16*, 253–266.
Li, J., Vasanthan, T., & Bressler, D. C. (2012). Improved cold starch hydrolysis with urea addition and heat treatment at subgelatinization temperature. *Carbohydrate Polymers*, *87*, 1649–1656.
Li, W., Corke, H., & Beta, T. (2007). Kinetics of hydrolysis and changes in amylose content during preparation of microcrystalline starch from high-amylose maize starches. *Carbohydrate Polymers*, *69*, 398–405.
Miao, M., Zhang, T., Mu, W., & Jiang, B. (2011). Structural characterizations of waxy maize starch residue following in vitro pancreatin and amyloglucosidase synergistic hydrolysis. *Food Hydrocolloids*, *25*, 214–220.
Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.
Nara, S., & Komiya, T. (1983). Studies on the relationship between water-satured state and crystallinity by the diffraction method for moistened potato starch. *Starch/Staerke*, *35*, 407–410.
Oates, C. G. (1997). Towards an understanding of starch granule structure and hydrolysis. *Trends in Food Science & Technology*, *8*, 375–382.
Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., & Johnson, D. K. (2010). Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels*, *3*, 1–10.
Planchot, V., Colonna, P., Gallant, D. J., & Bouchet, B. (1995). Extensive degradation of native starch granules by alpha-amylase from *Aspergillus fumigatus*. *Journal of Cereal Science*, *21*, 163–171.
Segal, L., Creely, J. J., Martin, A. E., & Conrad, C. M. (1962). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile Research Journal*, *29*, 786–794.
Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch—Composition, fine structure and architecture. *Journal of Cereal Science*, *39*, 151–165.
Thygesen, A., Oddershede, J., Lilholt, H., Thomsen, A. B., & Ståhl, K. (2005). On the determination of crystallinity and cellulose content in plant fibres. *Cellulose*, *12*, 563–576.
Wang, L., & Wang, Y. J. (2004). Rice starch isolation by neutral protease and high-intensity ultrasound. *Journal of Cereal Science*, *39*, 291–296.
Wang, L., & Wang, Y.-J. (2001). Structures and physicochemical properties of acid-thinned corn, potato, and rice starches. *Starch/Starke*, *53*, 570–576.
You, S., & Izydorczyk, M. S. (2007). Comparison of the physicochemical properties of barley starches after partial α-amylolysis and acid/alcohol hydrolysis. *Carbohydrate Polymers*, *69*, 489–502.
Zhang, Y. H. P., Cui, J. B., Lynd, L. R., & Kuang, L. R. (2006). A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: Evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules*, *7*, 644–648.
Zhou, Y., Hoover, R., & Liu, Q. (2004). Relationship between α-amylase degradation and the structure and physicochemical properties of legume starches. *Carbohydrate Polymers*, *57*, 299–317.
Zobel, H. F. (1988). Molecules to granules: A comprehensive starch review. *Stärke*, *40*, 44–50.