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Hydrolysis of concentrated raw starch: A new very efficient α-amylase from *Anoxybacillus flavothermus*

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ABSTRACT

A new α-amylase from *Anoxybacillus flavothermus* (AFA) was found to be effective in hydrolyzing raw starch in production of glucose syrup at temperatures below the starch gelatinization temperature. AFA is very efficient, leading to 77% hydrolysis of a 31% raw starch suspension. The final hydrolysis degree is reached in 2–3 h at starch concentrations lower than 15% and 8–24 h at higher concentrations. AFA is also very efficient in hydrolyzing the crystalline domains in the starch granule. The major A-type crystalline structure is more rapidly degraded than amorphous domains in agreement with the observed preferential hydrolysis of amylopectin. Amylose–lipid complexes are degraded in a second step, yielding amylose fragments which then re-associate into B-type crystalline structures forming the final α-amylase resistant fraction. The mode of action of AFA and the factors limiting complete hydrolysis are discussed in details.

1. Introduction

Starch and its two main constituents, amylose and amylopectin, are degraded by α-amylases which are the major enzymes involved in the hydrolysis of α(1→4) glycosidic bonds. α-Amylases are very important in biological reactions such as fermentation, germination or digestion, but also widely used in e.g. the industry for production of glucose syrups, control of anti-staling in bread products or in detergents to remove starch based stains.

As native starch is water insoluble at room temperature, many applications of amylases are carried out at high temperature and pressure where the starch is gelatinized. During gelatinization, the granular architecture and the molecular order (double helices) of the starch granule are disrupted. This change in the physical state is known to increase the susceptibility of starch to enzymatic hydrolysis (Lauro, Suortti, Autio, Linko, & Poutanen, 1993).

By contrast, the hydrolysis of solid starchy substrates strongly depends on starch structure and amylase source (Buleon, Colonna, Planchot, & Ball, 1998; Colonna, Leloup, & Buleon, 1992; Gallant, Bouchet, Buleon, & Perez, 1992; Gerard, Colonna, Buleon, & Planchot, 2001; Gernat, Radosta, Anger, & Damaschun, 1993; Oates, 1997; Williamson, Belshaw, Self, Noel, Ring, Cairns, Morris, Clark & Parker, 1992). The morphology and the surface of the granule, the amylose content, the crystalline structure or the presence of amylose-lipid complexes were shown to be limiting factors to hydrolysis of the starch granule.

While acid hydrolysis degrades preferentially the amorphous parts of the starch granule, α-amylases can solubilize both amorphous and crystalline domains (Colonna, Buleon, & Lemarie, 1988; Gerard et al., 2001). The mechanisms involved in the hydrolysis of the crystalline domains and especially the disruption of the double helices from the crystallite and their disentanglement are not well known. As double helices are too wide to enter the catalytic site of α-amylases (André, Buleon, Haser, & Tran, 1999), their disentanglement is speculated to occur during the adsorption stage. Adsorption of amylase onto starch granule was found to be a prerequisite for hydrolysis, but it can be inhibited by the presence of oligosaccharides such as maltose or maltotriose (Leloup, Colonna, & Ring, 1991). We recently studied a highly efficient fungal α-amylase from *Rhizomucor sp.* (RA) optimized for biofuel production, and able to degrade preferentially the crystalline fraction of maize starch (Tawil, Viksø-Nielsen, Rolland-Sabaté, Colonna, & Buleon, 2011). For this enzyme the slower degradation of amylose was attributed to the presence of lipid complexed amylose in the first stages of hydrolysis and then in the final stage to recrystallization of amylose fragments released by action of the amylase. Amylose-lipid complexes have proved to be present in cereal starches which naturally contain lipids (Morrison, Tester, Gidley, & Karkalas, 1993; Morrison, Tester, Snape, Law, & Gidley, 1993) but can also form when heating lipid containing starch in presence of water (Biliaderis, 1992;

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Buleon & Colonna, 2007; Le Bail, Bizot, Ollivon, Keller, Bourgaux, & Buleon, 1999). In the Vh-type, the most common crystalline form obtained by complexation of amylose with lipids, the single helices are packed in an orthorhombic unit cell with 16 water molecules within the unit cell (Rappenecker and Zugenmaier, 1981). The amylose–lipid complexes have also been shown to be more resistant to α-amylolysis than the A-type native structure present in cereal starches (Gernat et al., 1993; Tawil et al., 2011). However, hydrolysis is completed when an excess amount of enzyme or a longer hydrolysis time is applied (Biliaderis & Galloway, 1989; Holm et al., 1983).

In this study, we focus on a new bacterial α-amylase from *Anoxybacillus flavothermus* (AFA) found to be very efficient in hydrolysis of concentrated raw starch. This bacterial amylase contains a Starch Binding Domain (SBD) belonging to the CBM20 family and has been evaluated for use in low temperature glucose syrup production (Viksø-Nielsen, Andersen, Hoff, & Pedersen, 2006). The use of this enzyme compared to traditional high temperature α-amylases would require less energy and water than in the traditional liquefaction process. Its mode of action was studied in comparison to porcine pancreatic α-amylase (PPA) which has been widely studied for digestion of starch products and as model enzyme for hydrolysis of raw starch. AFA specificity was assessed by monitoring the evolution of starch structure especially morphology, crystalline structure and molar mass distribution during hydrolysis of maize starch at various concentrations. The limiting factors for a complete hydrolysis were also investigated.

2. Experimental

2.1. Materials

2.1.1. Substrates

Normal maize starch was from Cerestar (Cargill Vilvoorde, Belgium).

2.1.2. Enzymes

Purified preparation of AFA (Viksø-Nielsen et al., 2006) was provided by Novozymes, Denmark. Crystallized and lyophilized Porcine Pancreatic α-amylase and Proteinase K (from *Tritirachium album)* were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade.

2.2. Sample preparation

2.2.1. Enzymes

PPA was solubilized in 20 mM phosphate buffer, pH 6.5–7.0, containing 2 mM NaCl, 0.25 mM $CaCl_2$ and 0.2 g L^{-1} NaN_3. The solution was centrifuged at 4167 × *g* and the supernatant was used for hydrolysis after protein content determination. AFA was provided as 4 mg mL^{-1} solution in sodium acetate buffer. The concentration was determined by A280 and calculated using a molecular absorbance of 2.728 of the purified AFA, i.e. absorbance at 280 nm of a 1 mg mL^{-1} AFA solution.

2.2.2. Measurement of the enzyme activity

Enzyme activity was determined according to the Ceralpha procedure (McCleary et al., 2002). It consists to hydrolyze non reducing-end blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) by α-amylase, in the presence of excess α-glucosidase. The amount of released para-nitrophenol PNP was determined spectrophotometrically at a wavelength of 400 nm. One unit of activity (U) is defined as the amount of amylase required to release 1 μmole of PNP from BPNPG7 in one min at 40 °C, pH 7. Specific activities of AFA and PPA were 17 U and 3.5 U per mg of protein, respectively.

2.2.3. Sample preparation for high-performance size-exclusion chromatography coupled with multiangle laser light scattering and differential refractometric detection (HPSEC-MALLS-DRI)

Samples (10 mg) were pretreated in Me_2SO/H_2O (90/10 v/v), precipitated in 80% ethanol and dried. They were then solubilized in water by microwave heating under pressure (Rolland-Sabaté, Amani, Dufour, Guilois, & Colonna, 2003). The resulting solutions were filtered through 5 μm Durapore™ membranes. Carbohydrate concentration was determined by the orcinol sulfuric method (Planchot, Colonna, & Buleon, 1997). Sample recovery rates were calculated from the ratio of the initial concentration to the final concentration in solution.

2.3. Kinetics of α-amylolysis in heterogenous phase

α-Amylases from Porcine Pancreas (PPA) and *A. flavothermus* (AFA) were used at 37 °C, phosphate buffer pH 7 for PPA and 61 °C, acetate buffer pH 4.5 for AFA (resembling relevant application conditions). The following three starch concentrations were used: 5, 15 and 31% dry basis (d.b.), respectively. The amount of AFA and PPA was dosed at the same activity, i.e. 15.5 U per mg dry starch. Thus, 235, 710, and 1466 μL of AFA and 1.8, 5.5 and 11.4 mL of PPA were used for 5, 15 and 31% starch respectively, the final volume being adjusted to 20 mL with buffer. The suspension was shaken continuously in a water bath at 200 rpm and 2 mL aliquots were withdrawn at different time intervals (1, 2, 8, 24, 48, 72 and 96 h). For each aliquot, the reaction was stopped by adding 80 μL of 1 M KOH and then centrifuged at 4167 × *g* at 4 °C for 10 min. The precipitate was used for structural analysis. Total solubilized sugars were measured in the supernatant by the orcinol sulfuric method (Planchot et al., 1997), and the extent of hydrolysis was expressed as the ratio of soluble sugars from starch hydrolysis to the initial mass of starch (d.b.).

2.4. Analysis of the residual starch

2.4.1. Crystalline structure

X-ray diffraction (XRD) analysis was performed on native starch and residual starch withdrawn at different time intervals during hydrolysis. The water content of samples was adjusted by water phase sorption for 10 days in desiccators under partial vacuum at a relative humidity of 90% (using a saturated salt solution of baryum chloride). Hydrated samples (20 mg) were then sealed between two tape foils to prevent any significant change in water content during the measurement. XRD diagrams were recorded on a BRUKER™ (Wissembourg. France) D8 Discover diffractometer. Cu $K\alpha_1$ radiation (λ = 0.15405 nm), produced in a sealed tube at 40 kV and 40 mA, was selected using a Gobël mirror parallel optics system and collimated to produce a beam of 500 μm diameter. The diffracted beam was collected with a two-dimensional GADDS detector and recording time was 600 s. The distance from the sample to the detector was 100 mm. After normalization of all recorded diagrams at the same integrated scattering between 3 and 30° (2θ), relative crystallinity was determined as described previously (Maache-Rezzoug, Zarguili, Loisel, Queveau, & Buléon, 2008).

2.4.2. Molar mass distribution

The molar mass distribution of residues at different hydrolysis times was determined using high-performance size-exclusion chromatography (HPSEC) with multiple angle laser light scattering (MALLS) detection (Rolland-Sabaté, Guilois, Jaillais, & Colonna, 2011). Sample recovery was calculated from the ratio of the mass eluted from the column (integration of the refractometric signal) to the injected mass which was determined using the orcinol sulphuric method (Planchot et al., 1997).

2.5. Analysis of the soluble products

The composition of soluble products released by enzymatic hydrolysis was determined as previously described (Tawil et al., 2011) by using high-performance anion-exchange chromatography (HPAEC) with a pulsed amperometric detector (PAD) system (Pohu, Putaux, Planchot, Colonna, & Buleon, 2004). A detector response correction was performed for quantitative analysis using gluco-oligosaccharides from DP1 to DP7 (Sigma, Chemical Company).

2.6. Determination of the limiting factors for a complete hydrolysis

Potential inhibition of amylases by the end products was checked using washing of residual starch and a new 96 h hydrolysis after adding a new dosage of amylase solution as described previously (Tawil et al., 2011).

The concentration of amylase in the supernatant at the end of this new hydrolysis was determined according to the Bradford's procedure (Bradford, 1976) and the amount of adsorbed protein determined as $C = ((C_0 - C)/C_0) * 100$ with C_0 being the initial concentration. To check if the amylase adsorbed onto residual starch and/or aggregated at this stage was responsible for the hydrolysis rate decline, any starch bound enzyme was hydrolyzed by proteinase K. Thus, residual starch was washed one time in water and four times in Tris–HCL. Aliquot of proteinase K (0.015 mg per mg of starch) was then added onto each residual starch sample for 30 min. Proteinase K was removed by washing the residue 5 times with acetate buffer for AFA and with a phosphate buffer for PPA followed by centrifugation at 4167 × *g*. Subsequent hydrolysis was conducted as described above.

3. Results and discussion

3.1. Effect of raw starch concentration on the hydrolysis kinetics

Fig. 1 shows the evolution of hydrolysis kinetics for AFA with increasing starch concentration from 5 to 31% d.b. and Table 1 summarizes the corresponding values determined for AFA, and PPA after 96 h hydrolysis. No matter at what starch concentration tested, AFA is very effective on raw starch and surprisingly active on the 31% starch suspension since the final hydrolysis degree (FHD) reaches 77% for AFA, versus 32% for PPA after 96 h (Fig. 1 and Table 1). The hydrolysis degree decreases slightly when increasing the starch concentration with the FHD decreasing from 88 to 77% for 5 and 31% starch, respectively. All hydrolysis curves have a classical two phase shape but with a very rapid and short first stage which does not exceed 2–3 h for 5% and 8 h for 15% and 31% starch suspensions respectively (Table 1). Hydrolysis kinetics is much less dependent on starch concentration than for PPA. Even it is a little smaller than the values observed for RA (Tawil et al., 2011), the very high rate of hydrolysis and its extent at 2–8 h make AFA very interesting for rapid production of short chain maltodextrins from raw maize starch.

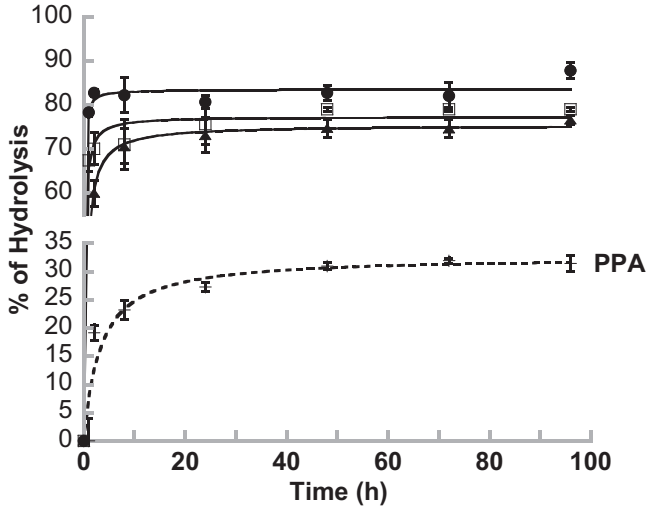

Fig. 1. Effect of raw starch concentration on AFA hydrolysis kinetics: 31% d.b. (▲) 15% d.b. (□), 5% d.b. (●). Data obtained for PPA (+) on 31% starch are shown for comparison.

3.2. Hydrolysis of the crystalline structure

The crystalline structure was essentially assessed by X-ray diffraction (XRD). Degree of crystallinity and crystalline type of residual starch are summarized, with the corresponding hydrolysis degree, in Table 2 for 5 and 31% starch suspensions at different times of hydrolysis. Native maize starch has A-type crystallinity with characteristic peaks at Bragg angle (2θ) = 15, 17, 18 and 23° (Fig. 2). The crystallinity decreases from 31% to 20% during the first 2 h of hydrolysis of the 31% starch suspensions by AFA (Table 2). The hydrolysis of crystalline domains is less pronounced in the 5% starch suspensions since a slightly lower decrease of crystallinity is observed while the hydrolysis extent is significantly higher (82% versus 60%). This ability to hydrolyse the crystalline domains at high starch concentrations is a unique feature of AFA, as for RA (Tawil et al., 2011), compared to more classical α-amylases as PPA for which the degree of crystallinity is kept constant all along the hydrolysis of 31% starch suspensions.

The intensity of the peak at 2θ around 20°, which corresponds to amylose–lipid complexes formed between amylose and endogeneous fatty acids present in maize starch (Vh-type), is remarkably stable at 31% starch. It develops more at 5% starch when the hydrolysis extent reaches more than 80% and is also present in the 96 h PPA residue obtained from 5% starch suspensions. Thus it appears that the Vh-type structure is much more resistant to α-amylases than the A-type, as already described by Gernat et al. (1993).

3.3. Changes in the macromolecular structure of starch during hydrolysis

The HPSEC-DRI traces of raw starch as well as AFA and PPA residual starch at 96 h (initial starch concentration 31%) are reported

Table 1
Kinetic data as a function of the starch concentration for AFA (and PPA in brackets).

Time (h)	5% db	15% db	31% db
1	78. ± 1 (25.0 ± 1.8)	67.4 ± 2.5 (22.0 ± 2.6)	51.8 ± 1.4 (19.0 ± 8.0)
2	82.7 ± 0.8 (35.0 ± 15.0)	70.1 ± 3.6 (29.0 ± 4.4)	60.0 ± 3.0 (23.0 ± 2.8)
8	82.2 ± 4.0 (50.0 ± 3.6)	71.0 ± 5.7 (34.0 ± 3.0)	70.6 ± 4.0 (27.0 ± 3.4)
24	80.7 ± 1.4 (66.0 ± 3.8)	75.7 ± 4.6 (45.0 ± 8.0)	73.1 ± 4.0 (18.4 ± 1.6)
48	82.8 ± 1.7 (78.0 ± 9.6)	79.0 ± 0.4 (48.0 ± 1.4)	74.7 ± 2.0 (32.0 ± 1.2)
72	82.0 ± 3. (84.0 ± 6.4)	79.0 ± 0.4 (54.0 ± 1.6)	74.6 ± 2.0 (31.5 ± 0.8)
96 (FHD)	87.9 ± 1.8 (89.0 ± 10.6)	79.0 ± 0.4 (67.0 ± 8.0)	76.6 ± 1.0 (32.0 ± 2.8)

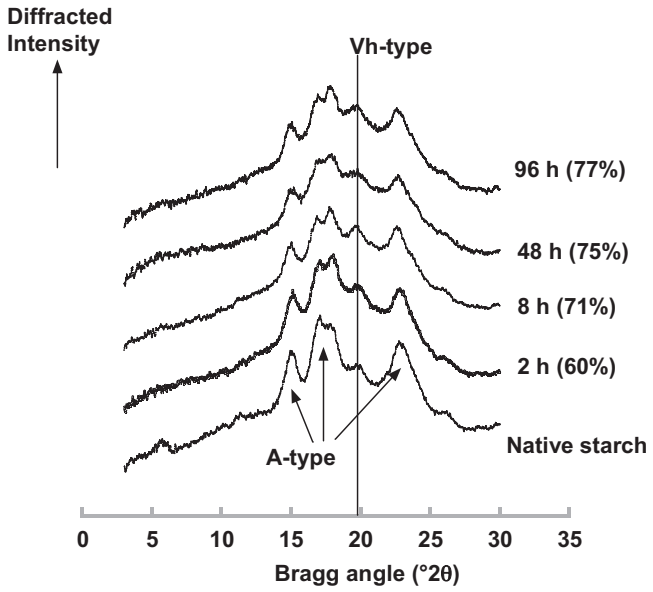

Fig. 2. Evolution of the crystalline structure as a function of time and hydrolysis degree (in brackets) during hydrolysis of a 31% starch suspension by AFA.

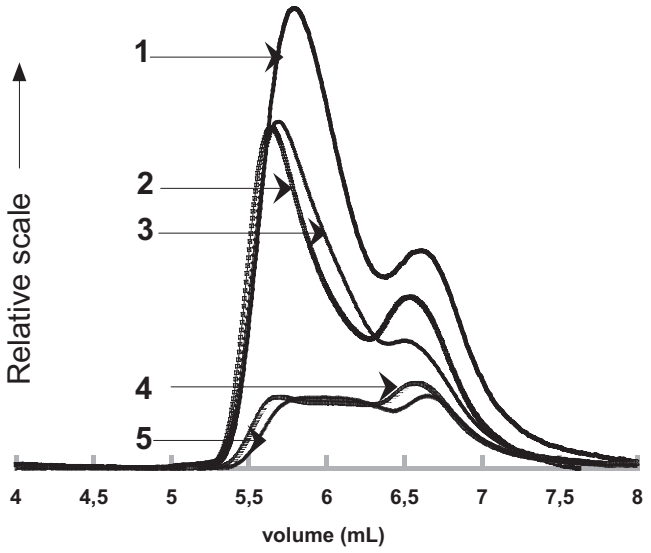

Fig. 3. HPSEC-DRI traces of residual starch after hydrolysis of 31% maize starch suspensions by AFA and PPA. 1: native starch (control); 2–3: residual starch obtained after hydrolysis with PPA at 48 h and 96 h respectively; 4–5: residual starch obtained after hydrolysis with AFA at 48 h and 96 h respectively. Relative scale was obtained by dividing each initial normalized profile (normalized refractometric response) by the amount of residual starch.

in Fig. 3. Raw starch presents two peaks, which are attributed to amylopectin (elution volume around 5.7 mL) and amylose (elution volume around 6.6 mL) according to previous work (Rolland-Sabaté et al., 2003). Amylopectin weight average molar mass decreases from 3.08×10^8 to 2.35×10^8 g mol^{-1} after 48 h. Structural information could be obtained by plotting the radius of gyration versus molar mass from the exponent ν_G according to the empirical power law: $R_G \sim M^{\nu G}$. The radius of gyration decreases with hydrolysis and ν_G, which was calculated on the whole amylopectin fraction, increases from 0.38 to 0.41 after 96 h of hydrolysis. ν_G value depends on the polymer shape, the temperature, and the polymer–solvent interactions: $\nu_G = 0.33$ for a sphere; $\nu_G = 0.5$–0.6 for a linear random coil, and $\nu_G = 1$ for a rod. The values determined here show that the amylopectin remaining fraction is slightly less dense in solution than native amylopectin and then probably slightly less branched. When looking at the chromatograms (Fig. 3) the amount of amylopectin has been more intensively reduced than that of amylose which is consistent with the decrease of crystallinity observed during hydrolysis. At the same time amylose is highly hydrolyzed, the weight average molar mass taken at the apex of the corresponding DRI peak being 2 times less after 96 h, decreasing from 5.65×10^6 to 2.58×10^6 g mol^{-1} (Table 3). Nevertheless, these latter values do not represent the absolute molar mass of amylose population since amylose and amylopectin peaks are not fully separated, and then low molar mass amylopectin fractions elute at the same elution volume as amylose ones. Moreover, a part of the amylose peak could involve partially hydrolyzed amylopectin eluting at the same volume. These results are in agreement with the observed large decrease of crystallinity during hydrolysis, since amylopectin is usually assumed to support the framework of the crystalline domains in the starch granule. In any case the behavior is different from that of PPA for which residual starch present the same bimodal shape as for native starch (Fig. 3), with no decrease of amylopectin weight average molar mass which is consistent with a granule per granule action mode as already proposed (Colonna et al., 1988).

Table 2
Evolution of the crystalline structure CS (degree of crystallinity in %, crystalline type A, B or Vh) and degree of hydrolysis HD (%) of maize starch during hydrolysis by AFA and PPA.

	Native starch	Hydrolyzed 2 h		Hydrolyzed 8 h		Hydrolyzed 48 h		Hydrolyzed 96 h	
	CS	CS	HD	CS	HD	CS	HD	CS	HD
AFA 5% starch	31, A	22, A + Vh	82	15, A + Vh	82	14, A + Vh	83	14, A + Vh	87
AFA 31% starch	31, A	20, A	60	20, A	71	15, A	75	15, A + Vh	77
PPA 5% starch	31, A	30, A	34	25, A	49	20, A	78	20, A + Vh	89
PPA 31% starch	31, A	30, A	23	30, A	27	30, A	32	30, A	32

Table 3
Weight average molar mass ($\overline{M_w}$), z-average radius of gyration ($\bar{R}_{GZ}$) and the slope of the log/log plot of molar mass versus radius of gyration (ν_G) of amylose and amylopectin after hydrolysis of maize starch (31% d.b.) by AFA; nd: not determined; (*) values obtained over the whole amylopectin peak; (+) values at the maximum of amylose peak; the experimental uncertainty was 5%.

	Amylopectin			Amylose	Amylose/amylopectin ratio
	$\overline{M_w}$ (g mol^{-1})*	$\bar{R}_{GZ}$ (nm)*	ν_G	$\overline{M_w}$ (g mol^{-1})+	
Native starch	3.08×10^8	270.0	0.38	5.65×10^6	0.5
AFA 48 h	2.38×10^8	236.0	0.44	1.92×10^6	nd
AFA 96 h	2.35×10^8	240.0	0.41	2.58×10^6	nd

Table 4
DP1 to DP7 composition in the soluble fraction during hydrolysis of 31% starch suspensions by AFA.

	Time (h)	DP1 (%)	DP2 (%)	DP3 (%)	DP4 (%)	DP5 (%)	DP6 (%)	DP7 (%)
AFA	2	14.1	34.2	16.5	2.9	17.6	9.1	5.6
	8	16.7	34.5	14.1	4.1	17.9	8.8	3.8
	48	19.0	35.3	9.5	5.6	18.7	8.2	3.7

3.4. Nature of the soluble oligosaccharides produced.

The distribution of oligosaccharides in the soluble fraction after 2, 8 and 48 h hydrolysis using AFA on 31% starch suspensions is shown in Table 4. Only the values obtained for DP (degree of polymerization) <8 are shown, since the HPAEC response is not quantitative above DP 7. The distribution is similar to the usual distribution obtained from hydrolysis by classical α-amylase as PPA (Pohu et al., 2004; Sharma, Yadav, & Ritika, 2008). The major oligosaccharide present in the soluble fraction is maltose (DP2), which is known to induce a potential inhibition of amylases (Colonna et al., 1992; Faisant et al., 1993). The composition changes slightly between 2 and 48 h (Table 4). The ratio DP1/DP3 and DP4/DP7 increases, evidencing a partial hydrolysis of DP3 into DP1 and DP7 into DP4 and DP3.

3.5. Limiting factors for complete hydrolysis

The relative activity of AFA as a function of time under the buffer and temperature conditions used for hydrolysis was determined. Under these conditions AFA loses its activity very rapidly and within 2–4 h. It could explain why an incomplete hydrolysis of starch was observed using this enzyme. Nevertheless, as the amount of hydrolyzed product slightly increases from 2 h to 8 h of hydrolysis at 5% starch and from 2 h to 24 h at higher starch concentrations, it was assumed that the substrate has a protective effect against AFA denaturation. A similar observation has been already reported in the literature (De Cordt, Hendrickx, Maesmans, & Tobback, 1994; Gorinstein, 1993). Starch is known to stabilize α-amylase activity at higher dry weight concentrations.

The degree of hydrolysis increases from 87 to 90% and from 77 to 82% for the 5% and 31% starch suspensions, respectively, after washing the residue and adding a fresh dosage of AFA. It is difficult to attribute this change to a slight inhibition by reaction products since AFA also lost its activity during the first hydrolysis.

No further hydrolysis was observed after removal of the adsorbed or aggregated AFA by proteinase K and adding a new amylase solution, i.e. indicating that no unproductive binding or aggregation of AFA prevents a complete hydrolysis, although less than 10% of enzyme was still present in the supernatant at the end of hydrolysis.

Thus, it was concluded that hydrolysis ends due to the presence of a residue resistant to AFA. The crystalline structure of this residue was analyzed by X-ray diffraction on the samples that were most hydrolyzed: 90% and 82% originating from 5 and 31% starch suspensions, respectively (after washing the residue and adding and fresh AFA as described above). As shown in Fig. 4, a mixture of A-+Vh-type is found in the 5% starch residue and almost pure B-type, with characteristic peaks at 2θ around 5.6, 17 and 24°, in the 31% starch residue hydrolyzed by AFA. It demonstrates that the mode of action of AFA is not the same on a 5 versus a 31% starch suspension. The resistance of the Vh-type at 5% starch could be due to improvement of the Vh crystals perfection by annealing at 61 °C. While at 31% starch, less water is available and annealing is less important. This explains the higher susceptibility of the Vh-type at 31% starch in comparison to 5% starch. The higher resistance to amylolysis of Vh-type when compared to A-type in cereal starches has been observed earlier (Gernat et al., 1993; Kwasniewska-Karolak, Nebesny, & Rosicka-Kaczmarek, 2008; Lauro, Forssell, Suortti, Hulleman, & Poutanen, 1999).

The B-type structure can originate from rearrangements of linear amylose-like fragments released by the enzyme. Such a rearrangement into B-type is favoured at higher starch concentrations. B-type structure is known to result from retrogradation of starch (Buleon & Colonna, 2007) or crystallization of short chain amylose in water (Buleon, Veronese, & Putaux, 2007). B-type structures have proved to be very resistant to hydrolysis (Colonna et al., 1992; Planchot et al., 1997). Such rearrangement during hydrolysis has already been described by Lopez-Rubio, Flanagan, Shrestha, Gidley, and Gilbert (2008) when looking at hydrolysis of high amylose starch. The B-type structure formed prevents any progress of hydrolysis, once the Vh-type is degraded.

4. General discussion

AFA is highly effective in hydrolysis of raw starch suspensions, being able to degrade 83% of a 5% and 60% of a 31% starch suspension respectively, within 2 h at 61 °C. The hydrolysis degree of a 31% starch suspension reaches 75–77% within 24 h. This very high efficiency at high starch concentration is remarkable. The hydrolysis degree observed on raw starch is very comparable to that determined for RA (Tawil et al., 2011) but is reached more rapidly, 70% for AFA versus 30% for RA after 8 h on a 31% starch suspension. This makes AFA more suitable for low temperature glucose syrup production. RA reaches similar extents of hydrolysis in 48 h but

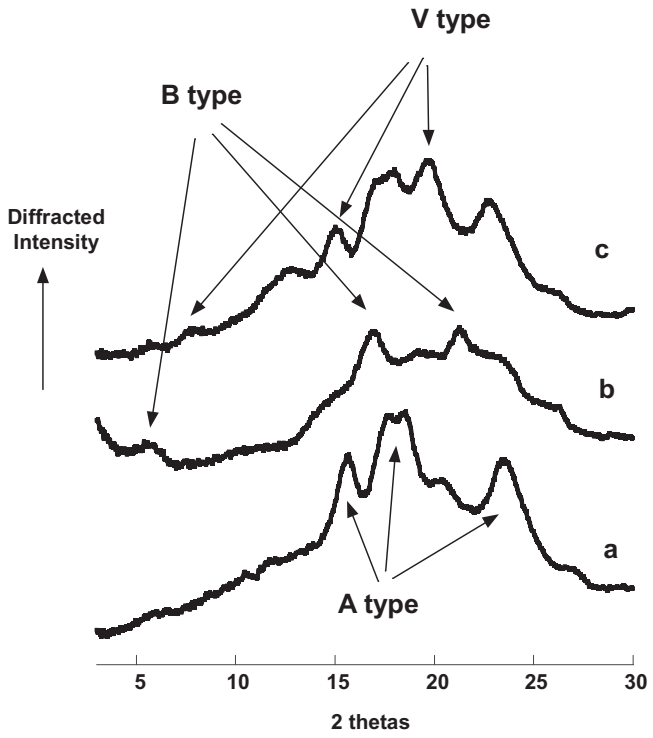

Fig. 4. Crystalline structure of (a) native maize starch (A type), (b) residual starch at 192 h hydrolysis of 31% starch by AFA (FHD 83%, B type) and (c) residual starch at 192 h hydrolysis of 5% starch by AFA (FHD 94%, A + Vh type).

was more suitable for bioethanol production since it works very efficiently at 32 °C and releases essentially glucose (Tawil et al., 2011).

The mode of action of AFA is very specific. Its efficiency is almost independent on starch concentration when compared to other α-amylases. Its behavior differs with changes in starch concentration since the residue from 5% starch consists of a mixture of residual A-type structure and a large amount of crystalline Vh-type amylose–lipid complexes while at 31% it consists of pure B-type structure. Therefore it seems that amylose-lipid complexes are more resistant to enzymatic attack at low starch concentration and that crystalline structures are more rapidly degraded at high starch concentration (Table 2). It differs from cellulases which usually degrade preferentially amorphous cellulose leading to an increase in the degree of crystallinity during hydrolysis (Zhang & Lynd, 2004). Moreover in this work the temperature used for hydrolysis (61 °C) may provoke some starch reorganization during hydrolysis as annealing or amylose–lipid complexing, especially when the α-glucan chains mobility increases after partial hydrolysis.

At 31% starch, amount of amylopectin is more intensively reduced than that of amylose at 48–96 h, which is consistent with both rapid hydrolysis of the crystalline domains, i.e. originating from the amylopectin, and the resistance to hydrolysis of lipid complexed amylose present in maize starch. Nevertheless, both total amount and average molar mass of amylose decrease also substantially which evidences hydrolysis of non complexed amylose. Indeed Morrison, Law, and Snape (1993c) showed that the rate of complexed amylose in native starch is about 15% of the total amylose content. In this work, the presence of Vh-type structure after AFA hydrolysis for 48 h and longer hydrolysis times could result from 2 mechanisms: (i) Vh-type crystalline domains could be present initially in the native granules but in a relative amount too small to be detected by X-ray diffraction before hydrolysis of the A-type structure. Moreover their crystallinity could be improved by annealing at 61 °C. (ii) Vh-type could be formed during hydrolysis between amylose fragments released by enzyme and lipid present in maize starch. By contrast, amylose and amylopectin amounts decrease concomitantly during PPA hydrolysis, which is consistent with a mode of attack granule by granule already observed for hydrolysis of wheat starch and other starches in solid state (Colonna et al., 1988; Oates, 1997).

The higher resistance of Vh-type structure, characteristic of lipid-complexed amylose, compared to A-type is controversial. The resistance has been observed during enzymatic hydrolysis of cereal starch at prolonged hydrolysis times by Gernat et al. (1993) and Lauro et al. (1999). In contrast Gerard et al. (2001) have shown on maize starch mutants that A- and Vh-types have the same susceptibility to PPA hydrolysis. Moreover, lipid-complexed amylose formed *in vitro* is completely degraded by α-amylases when an excess amount of enzyme or long hydrolysis time is applied (Biliaderis & Galloway, 1989; Faisant et al., 1993; Holm et al., 1983). Here, the temperature used for AFA assays was sufficiently high to favour either the annealing of existing complexes in native maize starch into a more crystalline structure or the formation of new complexes between amylose fragments released and lipid present in maize starch.

B-type structure appears at very high degrees of hydrolysis and at high starch concentration. It probably results from recrystallization of linear fragments of amylose released by the amylase when remaining amylose is widely hydrolyzed. The high initial concentration of starch favours crystallization and precipitation, and this type of structure probably constitutes the real resistant part to enzymatic hydrolysis. Such reorganization during enzymatic hydrolysis has already been observed by Lopez-Rubio et al. (2008) on resistant starch and high amylose maize starch and more recently on maize starch (Tawil et al., 2011). Recrystallization of amylose into B-type is also a major way to prepare highly resistant starch (Buleon & Colonna, 2007; Leloup, Colonna, & Buleon, 1991).

The very high efficiency of AFA for hydrolysis of raw starch, compared to that of PPA, is evidently due to the presence of a particularly important starch binding domain (CBM20) in this enzyme (Viksø-Nielsen et al., 2006). Such domain has been reported to facilitate adsorption of amylases on raw starch, the disentanglement of the double helices present in the crystalline lamellae and therefore the disruption of the starch structure results in a faster breakdown of the starch granules (Juge et al., 2006; Christiansen et al., 2009).

5. Conclusion

AFA is shown to be very efficient on concentrated raw starch suspensions with hydrolysis degrees reaching 60% after 2 h and 77% after 48 h for 31% starch. The resistance to hydrolysis is due to the presence of lipid-complexed amylose at low starch concentration, recrystallization of complexes being favoured at 61 °C and in excess water. Finally, at 31% starch rearrangement of amylose fragments into B-type structures is probably the final limiting factor for complete hydrolysis. One of the most surprising results is that AFA is able to hydrolyze the crystalline domains easier than the amorphous ones. The ability of AFA to hydrolyze preferentially crystalline structures at high starch concentration, as already observed for RA (Tawil et al., 2011), opens a new way in terms of fundamental approach of enzymatic hydrolysis in condensed systems but also for the design of new industrial processing of native starch.

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