



Functionality of porous starch obtained by amylase or amyloglucosidase treatments



A. Dura^a, W. Błaszczak^b, C.M. Rosell^{a,*}

^a Institute of Agrochemistry and Food Technology (IATA-CSIC), Avenida Agustín Escardino, 7, Paterna 46980, Valencia, Spain

^b Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland

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ABSTRACT

Porous starch is attracting very much attention for its absorption and shielding ability in many food applications. The effect of two different enzymes, fungal α -amylase (AM) or amyloglucosidase (AMG), on corn starch at sub-gelatinization temperature was studied as an alternative to obtain porous starch. Biochemical features, thermal and structural analyses of treated starches were studied. Microscopic analysis of the granules confirmed the enzymatic modification of the starches obtaining porous structures with more agglomerates in the case of AMG treated starches. Several changes in thermal properties and hydrolysis kinetics were observed in enzymatically modified starches. Hydration properties were significantly affected by enzymatic modification being greater influenced by AMG activity, and the opposite trend was observed in the pasting properties. Overall, results showed that enzymatic modification at sub-gelatinization temperatures really offer an attractive alternative for obtaining porous starch granules to be used in a variety of foods applications.

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1. Introduction

Starch is widely used in food and industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent (Singh, Kaur, & McCarthy, 2007). In general, native starches produce weak-bodied, cohesive, rubbery pastes when heated and undesirable gels when the pastes are cooled (Abbas, Khalil Sahar, & Meor Hussin, 2010). In order to meet its intended function, physical, chemical or enzymatic modifications are applied to achieve the functional properties not found in native starches (Jayakody & Hoover, 2002; Lacerda et al., 2008). The use of enzymatic modification has a number of advantages comprising fewer by-products, more specific hydrolysis products and high yield, besides better control of the process and end products with particular properties. There are many enzymes used to alter starch structure and to achieve desired functionality (Rosell & Collar, 2008). Enzymes hydrolyze (1 → 4) or (1 → 6) linkages between α -D-glucopyranosyl residues. The most common enzymes for starch modification include α -amylase, β -amylase, glucoamylase, pullulanase, and isoamylase. α -Amylase can hydrolyze the (1 → 4)- α -glucosidic bonds of starch in an endo-action. Hydrolysis occurs in a random fashion at any (1 → 4)-linkage within the starch chain to rapidly reduce the molecular size of starch and the viscosity of the starch solution during pasting. Amyloglucosidase is an

exo-acting enzyme that catalyzes the hydrolysis of both α -D-(1 → 4) and α -D-(1 → 6)-linkages from the non-reducing ends of the starch chain. Numerous researchers have investigated enzymatic hydrolysis of starches from cereals, roots, tubers, and legumes in terms of enzyme adsorption, action pattern, extent of hydrolysis, degree of crystallinity and hydrolysis products (Colonna, Leleup, & Buleon, 1992; Gallant, Bouchet, Buleon, & Perez, 1992; Hoover, 2001; Kimura & Robyt, 1996; Li, Vasanthana, Hoover, & Rossnagel, 2004). Taking into particular account that corn starch makes up more than 80% of the world market for starch, many researchers have been focused on the hydrolysis products released from the enzymatic reaction (Huang et al., 2010; Khatoon, Sreerama, Raghavendra, Bhattacharya, & Bhat, 2009; Li & Ma, 2011; Miao, Zhang, Mu, & Jiang, 2011). Therefore, kinetics mechanism of the enzymatic reaction and hydrolysis products have been studied by other researchers but the features of the modified starches have been the focus of a few studies. Simultaneous hydrolysis of waxy corn starch with amylase and amyloglucosidase have been reported for modifying the digestion of the starch (Miao et al., 2011), and reducing the digestibility of corn starch by partial α -amylase treatment (Han et al., 2006), or through the action of β -amylase or maltogenic α -amylase with transglucosidase (Ao et al., 2007). In addition, Lacerda et al. (2008) studied the thermal properties of corn starch treated with fungal α -amylase showing higher action on the amorphous region of the granule.

Lately, there is an increasing interest for the developing corn porous starch due to it has interesting properties for being used in the areas of food, medicine, chemical industry, cosmetics,

* Corresponding author. Tel.: +34 963900022; fax: +34 963636301.

E-mail address: rosell@iata.csic.es (C.M. Rosell).

agriculture and other fields. In fact, porous starch might be used in foods to ensure a steady release of spices, sweeteners, acid condiments, flavorings, or even to protect from light or oxygen highly oxidized compounds (Zhang et al., 2012). Porous starch is a modified starch that contains micro-sized pores on the surface and could be extended to the inner part of the granule. Previously, it has been reported its production by glucoamylase catalysis combined with ultrasonic treatment (Wu, Du, Ge, & Lv, 2011) and more recently Zhang et al. (2012) proposed the combination of α -amylase and glucoamylase optimizing the kinetic reaction for increasing the yield. Nevertheless, there is no information about the contribution of each enzyme to the starch changes. Because of modified starch is widely used in food formulations, it is of particular interest to determine biochemical features of starch and how they affect its functional properties. The aim of this research was to determine the independent effect of fungal α -amylase (AM) or amyloglucosidase (AMG) on corn starch at sub-gelatinization temperature, with special emphasis on biochemical features, thermal and structural analyses of treated starches.

2. Materials and methods

2.1. Materials

Corn starch samples were generously supplied by Huici Leidan (Navarra, Spain). The enzymes used were of food grade. Fungal α -amylase (AM) (Fungamyl 2500 SG) and amyloglucosidase (AMG) (amyloglucosidase 1100L) were provided by Novozymes (Bagsværd, Denmark). Chemical reagents from Sigma–Aldrich (Madrid, Spain) were of analytical grade.

2.2. Methods

2.2.1. Sample preparation

Preliminary assays were carried out for optimizing enzymatic reactions (starch quantity and pH), and pH 4.0 was selected for AMG reaction and pH 6.0 in the case of AM modification. The quantity of enzymes was based on previous experiments, where the amount of enzyme required to hydrolyze 50% of the starch (15%, w/v) at 95 °C for 10 min was selected (Dura, Calviño, Blaszcak, & Rosell, 2013).

Corn starch (5.0 g) was suspended in 25 mL of 20 mM NaH_2PO_4 buffer at pH 6.0 or in sodium acetate buffer at pH 4.0, those starch samples were referred as Control-6 or Control-4, respectively. For obtaining the enzymatic treated starches, enzymes (4 U of AMG/g starch and 5 U of AM/g starch) were added to the starch suspension. Samples were kept in a shaking water bath (50 rpm) at 50 °C for 24 h. Then, 50 mL of water were added and suspensions were homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, USA) during 1 min at speed 3. Samples were centrifuged for 15 min at $7000 \times g$ and 4 °C. Starches were washed again and centrifuged at the same conditions as before. Supernatants were pooled together and boiled in a water bath for 10 min to inactivate the enzymes before any further analyses (hydration properties and iodine binding values). Sediments containing starch were freeze-dried and kept at –25 °C for further thermal, biochemical and microstructural analyses. Four batches were prepared for each treatment.

2.2.2. Scanning electron microscopy (SEM)

The structural properties of the samples were studied using a JSM 5200 scanning electron microscope (JEOL, Tokyo, Japan). The corn starch powders were stick on a specimen holder using cuprum tape, and then coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). The obtained specimens were examined at an accelerating voltage of 10 kV.

The size of the holes induced by enzymatic action was measured using the image analysis program (ImageJ, UTHSCSA Image Tool software). Value was the average of 10 independent measurements. Eccentricity (e) was calculated for starch granules according to the Eq. (1):

$$e = \left[1 - \left(\frac{b^2}{a^2} \right) \right] \times \frac{1}{2} \quad (1)$$

where a corresponded to the larger diameter (length) and b to the shorter diameter (width) (Rojas, Rosell, Benedito, Pérez-Munuera, & Lluch, 2000). Values of eccentricity approaching 0 indicate very rounded structures, while values approaching 1 describe very elongated structures.

2.2.3. Starch content

Remaining free sugars on the treated starches were determined. Treated starch (0.1 g) placed in 10 mL Pyrex tubes was suspended in 2 mL of ethanol (80%) and incubated at 85 °C in a shaking water bath (50 rpm) for five min and then centrifuged ($1000 \times g$, 10 min, at room temperature). Supernatant was separated to measure free sugars released. This was performed for two times.

Damage starch was also determined by enzymatic method following the International Association for Cereal Chemistry standard method (ICC, 1996). The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) $\times 0.9$. Replicates ($n = 4$) were carried out for each determination.

2.2.4. Starch hydration properties and iodine binding values

Swelling parameters and water soluble compounds of modified corn starch samples were determined following the method reported by (Toyokawa, Rubenthaler, Powers, & Schanus, 1989), with slight modification as reported (Rosell, Yokoyama, & Shoemaker, 2011). Briefly, the supernatant was decanted into an evaporating dish and the weight of dry solids was recovered by evaporating the supernatant at 70 °C till constant weight. Four replicates were made for each sample. Residues (W_r) and dried supernatants (W_s) were weighed. Swelling power (SP), solubility index (SI) and swelling capacity (SC) were calculated as follows:

$$\text{Swelling capacity} \left(\frac{\text{g}}{\text{g}} \right) = \left(\frac{W_r}{W_i} \right) \quad (2)$$

$$\text{Solubility index} (\%) = \left(\frac{W_s}{W_i} \right) \times 100 \quad (3)$$

$$\text{Swelling power} \left(\frac{\text{g}}{\text{g}} \right) = \frac{W_r}{W_i} (100 - \text{SI}) \quad (4)$$

where W_i was the sample weight (g, db).

Iodine binding values are indicative of amylose complex formation. The iodine binding value was determined in the soluble supernatant. The soluble supernatant (40 μL) was mixed with 2 mL of an aqueous solution of 0.2% KI and 0.65% I_2 . The absorbance at 690 nm was measured using a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Paste clarity was directly measured in the supernatant as the absorbance at 650 nm using a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Values were the average from four replicates.

2.2.5. Pasting properties

The pasting properties were determined with rapid visco analyser (RVA) (Newport Scientific, model 4-SA, Warriewood, Australia) by following the American Association of Cereal Chemists Approved Method (AACC International, 1997), with some minor modifications. Distilled water (25 mL) was added to 2 g of corn

starch placed into the aluminum RVA canister. RVA settings during assessment were heating from 50 to 95 °C in 282 s, holding at 95 °C for 150 s and then cooling to 50 °C. Each cycle was initiated by a 10 s, 960 rpm paddle speed for mixing followed by a 160 rpm paddle speed for the rest of the assay. Viscosity was recorded during a heating–cooling cycle using Thermocline software for Windows (Newport Scientific Pvt. Limited, Warriewood, Australia).

2.2.6. Thermal properties

Thermal behavior from starch samples were determined using a differential scanning calorimeter (DSC) from Perkin–Elmer (DSC 7, Perkin–Elmer Instruments, Norwalk, CT), equipped with a thermal analysis data station (Pyris software, Perkin–Elmer Instruments, Norwalk, CT). For the study, corn starch samples were accurately weighed into aluminum DSC pans, and de-ionized water was added by micropipette to achieve a water-sample ratio of 2:1. The sample pans were sealed and equilibrated at room temperature for one hour before analysis. Nitrogen was used to purge analyses cells. Instruments were calibrated with indium, using an empty pan as reference. Thermal analysis consisted on heating from 30 to 120 °C at a rate of 10 °C/min. The onset temperature T_o , peak temperature T_p , and conclusion temperature T_c , were determined from the heating DSC curves. Gelatinization enthalpy (ΔH) was evaluated based on the area of the main endothermic peak, and peak height index (PHI) was calculated as $\text{PHI} = \Delta H / T_p - T_o$. All DSC experiments were replicated two times.

2.2.7. Starch hydrolysis kinetics

Starch hydrolysis was measured following the method described by (Gularte & Rosell, 2011) with minor modifications. Briefly, for free sugars removal, corn starch sample (0.1 g) suspended in 2 mL of 80% ethanol was kept in a shaking water bath at 85 °C for 5 min, and then centrifuged for 10 min at 1000 × g. The remaining pellet was incubated with porcine pancreatic α -amylase (6 U/mL) (Type VI-B, ≥ 10 units/mg solid, Sigma Chemical, St. Louis, USA) in 10 mL of 0.1 M sodium maleate buffer (pH 6.9) in a shaking water bath at 37 °C. Aliquots of 200 μ L were withdrawn during the incubation period and mixed with 200 μ L of ethanol (96%, w/w) to stop the enzymatic reaction and the sample was centrifuged at 10,000 × g for 5 min at 4 °C. The precipitate was washed twice with 50% ethanol (200 μ L) and the supernatants were pooled together and kept at 4 °C for further glucose enzymatic release.

Supernatant (100 μ L) was diluted with 850 μ L of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 50 μ L amyloglucosidase (33 U/mL) at 50 °C for 30 min in a shaking water bath. After centrifuging at 2000 × g for 10 min, supernatant was kept for glucose determination.

The glucose content was measured using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) × 0.9. Replicates ($n=4$) were carried out for each determination.

Experimental data were fitted to a first-order equation (Goni, Garcia Alonso, & Saura Calixto, 1997):

$$C_t = C_\infty(1 - e^{-kt}) \quad (5)$$

where C_t is the concentration of product at time t , C_∞ is the concentration at the end point, and k is the pseudo-first order rate constant.

2.2.8. Statistical analysis

Experimental data were statistically analyzed for analysis of variance (ANOVA) using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When analysis of variance indicated

significant F values, multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence. The correlation matrix was also performed using Statgraphics Centurion XV software.

3. Results and discussion

Enzymatic modification of the corn starch was carried out independently with α -amylase or amyloglucosidase under optimal pH conditions, 6.0 for α -amylase and 4.0 for amyloglucosidase. The action of each enzyme was compared with their specific control, which was submitted to the same treatment in the absence of enzymes. Results will reflect the effect of the pH and the enzymes on the starch features after being treated at sub-gelatinization temperature (50 °C). Zhang et al. (2012) found that 50 °C was the most suitable temperature for obtaining porous starch combining the action of AM and AMG. Also that temperature was useful for keeping the hydrolysis degree under control impeding the collapse and breakage of the granules that is produced during gelatinization.

3.1. Microstructure of the starch

To confirm the enzymatic action on the corn starch granules treated with α -amylase or amyloglucosidase, the microstructure was analyzed using SEM at two different magnifications 2000× (Fig. 1) and 3500× (Fig. 2) times. Corn starches without enzymatic treatment (Figs. 1a,c and 2a,c) showed that pH did not affect the morphology of starch granules. Granules appeared with round and polygonal granular shape, and their surface was relatively smooth with some hollows, attributed to the fingerprints of the native protein bodies. Huber and BeMiller (2000) explained that the crater-like impression resulted from pressing of protein bodies into the soft endosperm of the growing kernel. At high magnification some tiny cracks were visible. No swelling was observed in the native starch and the starch soaked at 50 °C for 24 h (results not shown). At high magnification (Fig. 2) it was observed that certain granules appeared pasted to each other, which was explained by the effect of some amylose leached out that acted as gluing material. No holes were visible on the control starches, indicating the absence of endogenous enzymes during the treatment period. Rocha, Felizardo, Jane, and Franco (2012) found that some holes could appear on the starch surface due to endogenous enzymes action during annealing.

The effect of enzymatic treatment was readily visible in the starches microstructure (Figs. 1b,d and 2b,d). Enzymatically treated starches showed changes in the surface but the shape of the granule hardly change. The microstructure of the starch granules was greatly dependent on the enzymatic treatment, but in both cases porous starch granules were obtained. α -Amylase produced some holes on the starch granules and its action was not dependent on the granule size. Amyloglucosidase was greatly active on the starch granules obtaining very perforated granules. At high magnification (Fig. 2b) different concentric layers could be distinguished within the holes. Huber and BeMiller (2000) described that in corn and sorghum starch granules, AMG produced openings to channels for providing access to the granule interior, and the surface pores enlarge through channels from hilum region toward the out-sides. In the present study, it seems that both enzymes attacked the starch granules on the fingerprint of protein bodies, which seems to be the weaker points more susceptible to enzymatic hydrolysis. In addition, AMG treated starches displayed a more stacked structure, with a kind of gel joining the granules. Likely, the polymers' chains leached out more easily during AMG enzymatic incubation and resulted in more abundant gel in the treated starch granules. Alternatively, it could be that the hydrolyzed amylose chains that

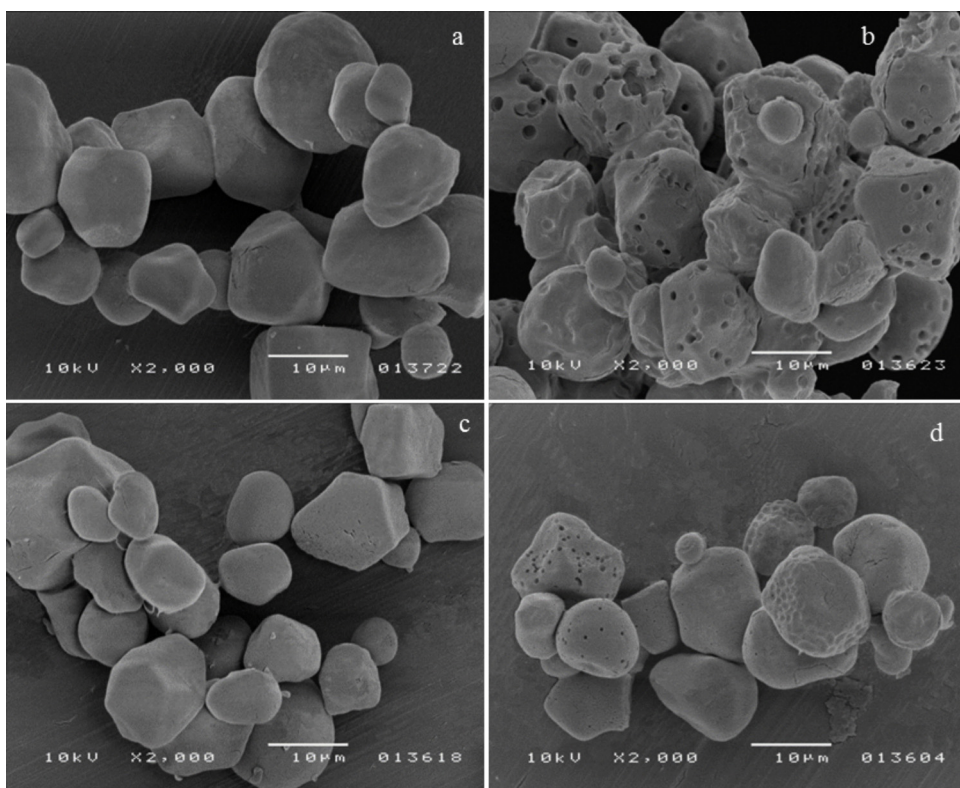


Fig. 1. Scanning electron micrograph of corn starch samples treated enzymatically (b and d) and their counterparts controls (a and c). Magnification 2000 $\times$. Control pH 4 (a); AMG (b); Control pH 6 (c); AM (d).

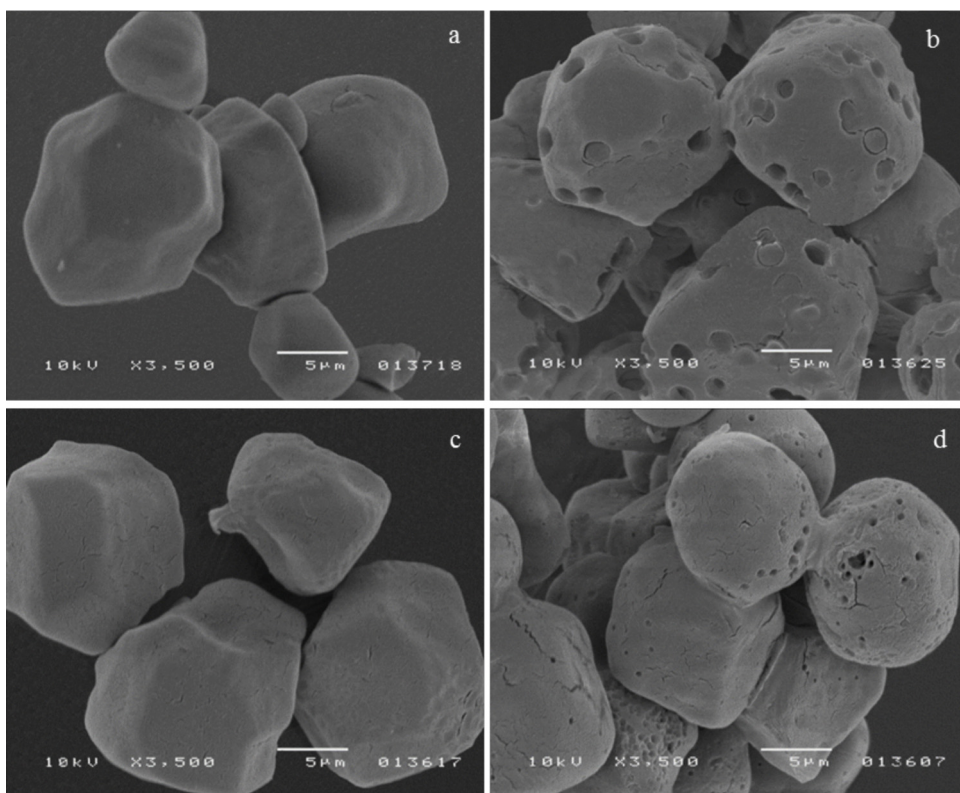


Fig. 2. Scanning electron micrograph of corn starch samples treated enzymatically (b and d) and their counterparts controls (a and c). Magnification 3500 $\times$. Control pH 4 (a); AMG (b); Control pH 6 (c); AM (d).

Table 1

Effect of enzymatic treatment on the hydration properties and chemical composition of the resulting porous starches.

	Control-4	AMG	Control-6	AM
Paste clarity (Abs 650 nm)	0.01 ± 0.01a	0.26 ± 0.03a	0.04 ± 0.01a	0.03 ± 0.00a
Solubility index (%)	1.13 ± 0.01a	20.08 ± 2.16c	1.27 ± 0.13a	4.3 ± 0.08b
Swelling power (g/g)	1.96 ± 0.12a	1.93 ± 0.00a	1.82 ± 0.01a	2.68 ± 0.15b
Amylose content (Abs 690 nm)	0.03 ± 0.01a	0.01 ± 0.01a	0.04 ± 0.02a	0.02 ± 0.01a
Swelling capacity (g/g)	1.94 ± 0.11b	1.54 ± 0.04a	1.80 ± 0.01b	2.56 ± 0.14c
Free sugars (mg/100 mg)	0.10 ± 0.01a	0.63 ± 0.15b	0.11 ± 0.03a	0.18 ± 0.09a
Damaged starch (mg/100 mg)	3.16 ± 0.24a	2.49 ± 1.22a	3.40 ± 0.24a	3.03 ± 0.97a

Mean ± standard deviation values followed by different letters within a column denote significantly different levels ($P < 0.05$) ($n = 4$).

leached out to the periphery of the granules were readily able to crystallize after cooling yielding the jelly structure.

The analysis of the size of the holes revealed that α -amylase treatment led to small holes of $0.15 \pm 0.04 \mu\text{m}$, whereas amyloglucosidase action resulted in bigger holes ($1.72 \pm 0.18 \mu\text{m}$) and the eccentricity ranged from 0.10 to 0.13, indicating very rounded pores in both cases.

It is generally accepted that granules contain amorphous and crystalline domains arranged in alternating concentric rings that create a semicrystalline environment within the granule (Ratnayake & Jackson, 2008), which were only evident on the AMG treated starch granules. Considering that the crystalline domains are mainly composed of amylopectin while bulk amorphous domains are made up of amylose traversed by non-crystalline regions of amylopectin, it might be expected that treatment at 50°C promotes changes in the amorphous areas of granules (Ratnayake & Jackson, 2008), leading to more structured internal structure.

3.2. Assessing the enzymatic treatment of the corn starch

After the enzymatic treatment, some information about the released compounds was gathered to confirm again that the enzymatic modifications occurred on the starch granules. Because of that, paste clarity, solubility index, swelling power, and apparent amylose content were measured (Table 1). Considering the values obtained for the control at pH 4.0 and 6.0, it was observed that pH did not significantly affect those parameters. Paste clarity, related to the compounds leached out during enzymatic treatment, was only increased with the AMG treatment, which can be explained by the excision activity of this enzyme that hydrolyze α -1,4- and α -1,6-glycosidic linkages from non-reduced extremes releasing free sugars. Therefore, it was expected relatively higher amount of residual free sugars. In fact, the solubility index value was significantly ($P < 0.05$) enhanced by AMG, followed by AM. Solubility index represents the amount of water soluble products obtained from the treated starch. AMG acted breaking the degree of association between intermolecular bonds (Dura et al., 2013), and more soluble compounds were leached. In opposition, it seems that the hydrolysis products released from α -amylase action were less soluble in water. Amylose that leached to the supernatant during incubation and enzymatic treatment was evaluated, but neither the pH nor the enzymatic modification of corn starch did cause changes in the apparent amylose content of the starches. These results indicated that the amount of leached amylose in the supernatant after thermal treatment at 50°C was insignificant, and that gelatinization of the starch granules did not occur during the process, which agrees with previous results (Rocha et al., 2012). Taking into account that no differences were observed on the apparent amylose content, thus water soluble compounds but no amylose chains were released during enzymatic treatment.

Swelling power slightly increased in enzymatically modified samples from control samples. Swelling power and solubility can provide evidence in assessing the extent of interaction between starch chains within the amorphous and crystalline domains of the

starch granule (Li, Shu, Zhang, & Shen, 2011; Ratnayake, Hoover, & Warkentin, 2002).

The enzymatically treated starches were washed to remove the remnant of any hydrolysis product and the swelling capacity of the porous starch granules as well as the amount of damage starch was determined (Table 1). The amount of free sugars was also quantified in the starch granule samples in order to verify the absence of hydrolysis products. The level of free sugars in the samples was very low ($<1\%$). Thus, the washing carried out to remove the released products after hydrolysis was effective, although AMG treated starch had more residual sugars than its respective control. Damage starch content did not differ among samples. Presumably, enzyme treatment modified the structure of corn starch enough to permit the water intake but not the rapid enzyme absorption and penetration into the starch structure, required in the measurement procedure of damage starch. According to (Biliaderis, 1991), the crystalline areas of starch maintain the structure of the granule, control its behavior in the presence of water and make it more or less resistant to chemical and enzymatic attack. The amorphous zone of starch granules is the least dense, is more susceptible to enzymatic attacks and absorbs more water at temperatures below the gelatinization temperature (Zavareze & Dias, 2011).

Starch granule swelling is known to begin in the bulk relatively mobile amorphous fraction and, in the more restrained amorphous regions immediately adjacent to the crystalline region (Donovan, 1979). Factors such as temperature, pH and type of enzyme can greatly influence the starch structure. When starch granules are heated in the presence of water, the starch granules absorb water and swell but since the enzymatic reaction occurs at 50°C , below gelatinization temperature, the water absorption was rather low. Nonetheless, the porous structures obtained by enzymatic treatments showed opposite behavior pertaining swelling capacity, AMG reduced the swelling capacity of the starch granules, in contrast with the increase obtained in the AM treated starches. During annealing, molecules in the amorphous regions of the granule hydrate and increase the mobility of amorphous area, and cause a limited but reversible swelling of the granule (Perry & Donald, 2000). The annealing results in a more perfectly ordered structure and in an increase in the granule stability by improving the arrangement of double helices, as well as the perfection of starch crystallites (Jayakody & Hoover, 2008). Plausible explanations could be either the enzyme action on the granule surface seems to release the more accessible compounds that bind water molecules or the agglomerates structure reduces the surface area that might be in contact with water molecules.

3.3. Pasting properties

The pasting properties of the porous starches were determined following a heating-cooling cycle (Fig. 3). Plots of the pasting portraits showed minor dissimilarities than expected taking into account the great microstructure divergences earlier mentioned. The viscosities of the control starches subjected to different pHs were initially overlapped indicating similar absorption. This agrees

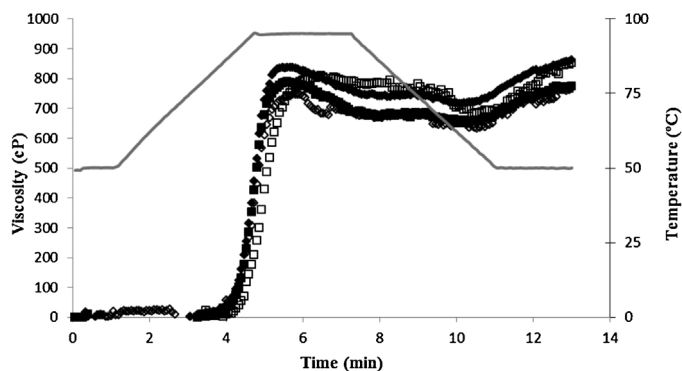


Fig. 3. RVA profiles of the corn starches treated with amylase ($\diamond$ or amyloglucosidase ($\square$) compared with their respective controls (without enzymatic treatment) in closed symbols (pH 4.0, $\blacksquare$; and pH 6.0, $\blacklozenge$).

with reported results obtained for swelling capacity, but also alike swelling and gelatinization rate (Sandhu & Singh, 2007). The maximum viscosity reached after heating was higher at pH 6.0, and then higher viscosity was observed along the cooling stage. Therefore, pH during incubation affected the annealing process that might take place during incubation at 50 °C, and lower pH caused larger changes than pH 6.0. Regarding the enzymes action, AMG treated starch showed a delayed pasting formation, likely water absorption and swelling ability of this starch was somewhat hindered after the enzymatic treatment, which was also observed when the hydration ability of the starch was determined. After gelatinization was completed, the AMG treated starch showed higher maximum viscosity, higher cooking stability and viscosity after cooling. Conversely, the AM treated starch showed similar swelling and hydration than its control counterpart, but displayed lower maximum viscosity after cooking and cooling, which has been related to the reduction of the molecular size of starch (Miao et al., 2011).

Parameters recorded from the pasting curves are shown in Table 2. The onset temperature, the temperature where viscosity first increased, was rather similar in the starches kept at different pHs. The trend observed was the same as the one followed by the swelling capacity, which might be expected since the initial stage of the gelatinization is the water absorption and in consequence the starch swelling. Values of the onset temperature for the enzymatically treated starches confirmed that AMG treated starch swelled slowly compared to its counterpart control. Hydrolysis percentage induced by each enzyme was calculated referred to the maximum viscosity given by a known amount of starch obtained after heating and cooling. AM promoted a 11% hydrolysis, but AMG did not decrease the viscosity, despite the porous structure of the modified starch. The reduction in the viscosity led by AM was kept beyond heating and cooling. Therefore, although microstructure appearance of AM treated starch seems to be more intact than the one of AMG treated starch, pasting behavior suggested that AM affected the loosely packed internal region of the granules and in lesser

Table 2
Effect of enzymatic treatment on the pasting parameters of corn starch.

	Control-4	AMG	Control-6	AM
Onset temp (°C)	83.2b	86.4c	82.5a	83.2b
Peak time (min)	5.6a	6.5b	5.5a	5.7a
Peak viscosity (cP)	788a	809b	838c	747a
Trough (cP)	650a	681b	715c	633a
Breakdown (cP)	138b	128a	123a	114a
Final viscosity (cP)	773a	853b	861b	767a
Setback (cP)	123a	172c	146b	134ab
Hydrolysis 95 °C (%)	0	0	0	11
Hydrolysis 50 °C (%)	0	0	0	11

Table 3

Thermal properties of enzymatically modified corn starches determined by DSC.

	Control-4	AMG	Control-6	AM
T_o (°C)	61.43a	67.48d	64.71b	66.54c
T_p (°C)	71.37b	72.18c	71.36b	70.43a
T_c (°C)	75.00a	77.00c	76.00b	75.00a
$T_p - T_o$	9.94d	4.70b	6.65c	3.89a
ΔH (J/g)	28.24b	20.40a	28.04b	29.87c
PHI (J/g °C)	2.84a	4.34b	4.22b	7.68c

T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, ΔH = enthalpy change, PHI = peak high index.

Values followed by different letters within a column denote significantly different levels ($P < 0.05$) ($n = 4$).

extent the densely packed periphery. Miao et al. (2011) studying the digestion of waxy corn starch with a combination of pancreatic α -amylase and amyloglucosidase found that the end distant from the hilum of native cereal starch was more susceptible to amylolysis, and empty shells were obtained when the hydrolytic reaction progressed in major extent. Nevertheless, although these authors proposed that hydrolytic enzymes act primarily through surface erosion of the starch granules, the results obtained with AM in the present study seem to indicate that AM exerts its major action in the inner core and only small pinholes are necessary for entering.

The opposite trend was observed with AMG that increased the viscosity of starch granules, compared to its control at pH 4.0, and gave lower breakdown related to the cooking stability of the starch granules during heating (Rojas, Rosell, & Benedito, 2001). In addition, this porous starch showed higher setback, related to amylose chains recrystallization, which suggested that higher amount of amylose chains leached out of the treated granule and they were rapidly able to form helical structures responsible of the gel formation.

3.4. Thermal properties

Thermal properties of the enzymatically modified corn starches were determined by differential scanning calorimetry (DSC) to detect possible changes in physical states of the starch structures (Table 3). The transition temperatures (T_o , T_p and T_c), gelatinization temperature range ($T_c - T_o$), enthalpies of gelatinization (ΔH) and peak height index (PHI) significantly ($P < 0.05$) differed among samples. Gelatinization temperature (T_p) of the corn starch was 71.4 °C, which was higher than the 64–69 °C previously described (Zhang et al., 2012; Rocha et al., 2012). Nevertheless, the increase could be ascribed to the annealing process that took place during incubation at 50 °C that produces an increase in the peak temperature (Rocha et al., 2012). Differences were observed in the thermal parameters derived from the pH effect; gelatinization temperatures were sifted to higher values, with exception of the T_p , when increasing the reaction pH. The effect was even more accentuated when compared the gelatinization temperature range at both pHs; pH 6.0 led to narrow gelatinization range, that has been related to major proportion of crystalline region (Zhang et al., 2012). It is well known that during the endothermic transition, namely gelatinization, primarily water molecules freely diffuse into the amorphous region of starch and secondly they penetrate the crystalline region (Biliaderis, 1991). Since differences were mainly observed on the T_o , it might indicate that amorphous region was more affected at higher pH during incubation.

Enzymatic treatment significantly modified the thermal properties of corn starch, delaying the gelatinization process, which started at higher onset temperature (Table 3). Starch modified with AMG showed the highest value of T_o (67.48 °C) compared to its control (61.43 °C), therefore higher gelatinization temperature is required to initiate gelatinization of the starch, which agrees with

previous observations described in the pasting behavior. The overall effect of enzymes on the gelatinization temperatures resulted in significantly lower gelatinization temperature range, mainly in the case of AM. That result was due to a delay in the start of gelatinization more than a shift of the conclusion temperature. α -Amylase is a very common endoenzyme that cannot hydrolyze either the α -1,6-glycosidic bonds that form the branch points in amylopectin, or the α -1,4-glycosidic bonds that are in close proximity to a branch point. Higher transition temperatures in the AMG samples have been explained due to higher degrees of crystallinity, which provide structural stability and make the granules more resistant to gelatinization (Kaur, Singh, Ezekiel, & Sodhi, 2009). Despite the different catalytic mechanism of both enzymes, it seems that the modification of starch induced by enzymes mainly affected the swelling behavior, which initially governs the gelatinization.

The higher ΔH of AM enzymatically modified corn starch compared to its respective control suggested that the state of the crystalline and amorphous regions differed from those of the AMG corn starch, in which the gelatinization enthalpy decreased in comparison with the control sample at pH 4. The lower ΔH values indicated that hydrolyzed starch by AMG required less thermal energy for gelatinization compared to the other samples. Gelatinization involves the uncoiling and melting of the external chains of amylopectin that are packed together as double helices in clusters (Cooke & Gidley, 1992). Crystallinity of starch is caused essentially by amylopectin polymer interactions, with the outer branches of amylopectin molecules interacting to arrange themselves into “crystallites” forming crystalline lamellae within the granule (Tester, Karkalas, & Qi, 2004). Studies carried out on granular starch and model crystallites confirmed that ΔH is mainly due to the disruption of the double helices rather than the long range disruption of crystallinity (Cooke & Gidley, 1992). Therefore, AMG action affected the double helical conformation of the granule, both amorphous and crystalline domains, and likely acting on the amylopectin side chains, due to amylopectin plays a major role in starch granule crystallinity and the presence of amylose lowers the melting point of crystalline regions and the energy for starting gelatinization (Sodhi & Singh, 2003). Conversely, α -amylase acted mainly on the amorphous regions leading to a more crystalline structure that requires higher gelatinization enthalpy.

Values of peak height index (PHI), a measure of uniformity in gelatinization, were clearly influenced by the pH used during the incubation of starch. Again the lower pH (4.0) was more aggressive on the starch granules, giving lower PHI, which suggests narrowed transition range for gelatinization (Krueger, Walker, Knutson, & Inglett, 1987). Compared with their respective control starches, enzymatically modified corn samples showed higher PHI values, indicating differences in the granule structure.

Overall, the low transition temperatures of the enzymatically treated starches indicates the reduction in the amorphous domain of the granules, which was accompanied by changes in the crystallite regions following a cooperative process in the case of AMG, but led to more crystalline structures in the AM treated starches.

3.5. Starch hydrolysis kinetics

Even though corn starches are not consumed directly but after being subjected to different processes, the enzymatic *in vitro* hydrolysis was carried out with the aim to determine the susceptibility of those enzymatically treated starches, with a more porous structure, to the enzymatic digestion. The digestibility curves of the enzymatically treated starches besides their respective controls are displayed in Fig. 4. No differences could be envisaged due to the pH difference between the control starches; only at the very end of the digestion reaction was observed that starch kept at pH 4.0 showed higher hydrolysis. In fact, the reaction rate

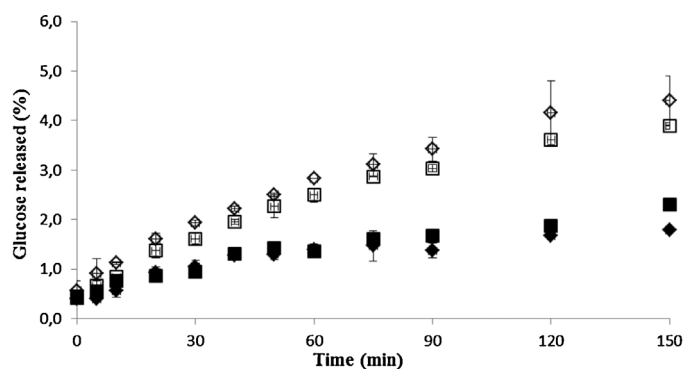


Fig. 4. Enzymatic starch hydrolysis of the corn starches treated with amylase ($\diamond$) or amyloglucosidase ($\square$) compared with their respective controls (without enzymatic treatment) in closed symbols (pH 4.0, $\blacksquare$; and pH 6.0, $\blacklozenge$).

calculated as the slope of the very early hydrolysis stage (0–30 min), showed similar values ($0.036 \text{ mg } 100 \text{ g}^{-1} \text{ s}^{-1}$). Again, low pH seems to affect in greater extent the granule structure. It is clearly evident that enzymatically modified corn starches showed greater susceptibility to be digested. Nontreated starch granules offer great resistance to enzymatic hydrolysis, thus the possible damage suffered by starch granules due to enzymatically treatment was clearly visible in the digestion. The increase in the enzymatic hydrolysis suggested that the starch granules became more accessible to enzyme hydrolysis. It has been reported that the presence of pores on the surface of annealed granules favored the action of endogenous amylases during annealing, contributing to a greater exposure of the amorphous areas to the exogenous enzymes (Rocha et al., 2012; Zavareze & Dias, 2011). In the present study, the enzymes used for obtaining the enzymatically treated starches originated porous granules with diverse openings size, which seems to accelerate the enzymatic digestion, and that effect was even greater in the case of AM treated starches. In fact, the reaction rate at early stage was for AMG-starch and AM-starch $0.054 \text{ mg } 100 \text{ g}^{-1} \text{ s}^{-1}$ and $0.067 \text{ mg } 100 \text{ g}^{-1} \text{ s}^{-1}$, respectively. The pancreatic α -amylase affinity for digesting starches is dependent on the particle size of starch, due to the enzyme feasibility for binding/absorption and the degree of order of starch has important influence on the initial rate at which native starch is digested by amylase (Tahir, Ellis, & Butterworth, 2010).

Starch hydrolysis curves were plotted to obtain the rate constant fitting the values to a first-order equation. Table 4 shows that the pseudo-first order rate constant estimated by the first order model described by Goni et al. (1997). Significant ($P < 0.05$) differences were found on the digestibility constant (k) due to the pH, showing higher rate constant the starch kept at pH 6.0. It has been described that significant differences in k are indicative of structural differences (Butterworth, Warren, Grassby, Patel, & Ellis, 2012), which agrees with previous observation encountered on pasting and thermal properties. Regarding the enzymatic treatment, porous starches showed lower digestibility constant compared to their respective controls and no differences were observed pertaining the enzyme hydrolytic action. It has been proposed that low k values are related to a slow diffusion of pancreatic amylase into the starch granule as digestion proceeds (Dhital, Shrestha, & Gidley,

Table 4

Kinetic parameters extracted from first-order fitting of the experimental enzymatic hydrolysis of modified corn starches.

	Control-4	AMG	Control-6	AM
$k \text{ (min}^{-1}\text{)}$	0.027 ± 0.009	0.017 ± 0.001	0.039 ± 0.002	0.017 ± 0.003
$C_{\infty} \text{ (%)}$	1.939 ± 0.150	4.114 ± 0.219	1.607 ± 0.065	4.592 ± 0.195

Mean $\pm$ standard deviation values ($n = 4$).

2010), although considering the microstructure of the porous granules the most plausible explanation to those values of rate constant seems to be the substrate exhaustion (Butterworth, Warren, & Ellis, 2011).

The percentage of enzymatic hydrolysis from the samples increased progressively with time during incubation. As described Butterworth et al. (2012), the rate of reaction decreases as the time is extended and plots of the concentration of product formed (or quantity of starch digested) against time are logarithmic. The plots approach an end point where no further reaction is measurable no matter how much longer incubation times are prolonged. This response is predictable based on the assumption that the concentration of available starch substrate decreases with time as starch is converted to products.

The end point values (C_{∞}) obtained in the hydrolyzed process reflected the concentration at the equilibrium point (Table 4). Results were significantly different, being affected by pH and enzyme treatment. Starch kept at pH 4.0 reached higher C_{∞} than that at pH 6.0, indicating again that low pH during annealing affected microstructure of the granule. Enzymatically modified corn starches resulted in higher C_{∞} values compared to their respective controls. Higher concentration of final product reflected increased digestibility of starch granules. AM treated starch showed higher C_{∞} than AMG treated starch. Therefore, AM action led to more accessible granules that were easily digested.

4. Conclusion

Demands of modified starches are increasing in parallel to the rapid development of food industry. This study showed that enzymatic modification of corn starch by α -amylase or amyloglucosidase at sub-gelatinization temperatures led to porous starch granules that differed in both, the microstructure surface and the internal morphology. Results confirmed that the loss of granular structural order and changes in both amorphous and crystalline domains during sub-gelatinization temperatures can be more influenced by the amyloglucosidase than α -amylase, and changes increased the susceptibility of starches to be digested.

Overall, results showed that enzymatic modification at sub-gelatinization temperatures really offer an attractive alternative for obtaining porous starch granules to be used in a variety of foods applications. The degree of porosity could be controlled by using different enzymes or a combination of enzymes might be considered as well.

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