Comparison of porous starches obtained from different enzyme types and levels

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Abstract

The objective was to compare the action of different hydrolases for producing porous corn starches. Amyloglucosidase (AMG), α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase) and branching enzyme (BE) were tested using a range of concentrations. Microstructure, adsorptive capacity, pasting and thermal properties were assessed on the porous starches. SEM micrographs showed porous structures with diverse pore size distribution and pore area depending on the enzyme type and its level; AMG promoted the largest holes. Adsorptive capacity was significantly affected by enzymatic modification being greater influenced by AMG activity. Unexpectedly, amylose content increased in the starch treated with AMG and BE, and the opposite trend was observed in AM and CGTase treated samples, suggesting different mode of action. A heatmap illustrated the diverse pasting properties of the different porous starches, which also showed significant different thermal properties, with lower To and Tp. Porous starch properties could be modulated by using different enzymes and concentrations.

Keywords: Porous starch, amyloglucosidase, α-amylase, CGTase, branching enzyme, microstructure.

1. Introduction

Porous starches are now attracting much attention due to their great adsorption ability (Zhang, Cui, Liu, Gong, Huang & Han, 2012). Those starches contain abundant pores from the surface to the center of the granules, which increase the specific surface area, acting as excellent natural absorbents. In fact, there is a growing interest in exploiting their properties in different food and non-food areas. In food industry, porous starches are used as colorants, spices, flavorings, sweeteners carriers and also for protection of sensitive elements such as oils, minerals, vitamins, bioactive lipids, food pigments such as β-carotene and lycopene which are sensitive to light, oxidation or high temperature (Belingheri, Giussani, Rodriguez-Estrada, Ferrillo & Vittadini, 2015; Luo et al., 2013; Majzoobi, Hedayati & Farahnaky, 2015).

Enzymatic treatments have been performed for obtaining porous starches, mainly applying glucoamylases and α-amylases (Sujka & Jamroz, 2007). Cassava starch granules were treated with α-amylase from *Bacillus amyloliquefaciens* without altering the size or morphology of the granules but significantly changing their properties (Ichihara, Fukuda, Takaha, Yuguchi & Kitamura, 2013). The combination of glucoamylase and α-amylase has been also proposed due to their synergistic action to hydrolyze raw starch completely very rapidly (Sun et al., 2010). In fact, porous starch was obtained using a combination of α-amylase and glucoamylases activity after optimizing the kinetic reaction to increase the reaction yield (Zhang, Cui, Liu, Gong, Huang & Han, 2012). Later, Dura, Błaszczak and Rosell (2014) compared the α-amylase and glucoamylase individual action to determine their effect on biochemical features, thermal and structural properties of corn starch. Researchers concluded that α-amylase or amyloglucosidase when acting on corn starch at sub-gelatinization temperatures for 24 or 48 hours led to porous starch granules that differed in both, the

51 microstructure surface and the internal morphology. Similarly, Chen (2012) hydrolyzed
52 native corn starch granules using glucoamylase at 50 °C for 1-8 h studying the impact of
53 enzyme/granule ratio and hydrolysis time on the microstructure of porous starch.
54 Research carried out on enzymatic treatments of starches has been accomplished using
55 diverse enzymes and experimental conditions (Sorndech et al., 2016; Uthumporn,
56 Zaidul & Karim, 2010), which complicates results comparison and a real understanding
57 of the enzymes action on the structure and functionality of the starches.
58 In addition, other starch acting enzymes like α-glucanotransferases have received
59 considerable attention to remodel parts of the amylose and amylopectin molecules by
60 cleaving and reforming α-1,4- and α-1,6-glycosidic bond (van der Maarel & Leemhuis,
61 2013) or in the case of cycloamylose glucanotransferase for producing cyclodextrins
62 (CDs) (Yamamoto, Zhang & Kobayashi, 2000). Nevertheless, α-glucanotransferase
63 such as branching enzyme or the cycloamylose glucanotransferase have been not tested
64 for obtaining porous starches.
65 The aim of this study was to compare the effect of different enzymes on corn starch
66 properties, taking also into account the impact of enzyme level. Amyloglucosidase
67 (AMG), fungal α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase) and
68 branching enzyme (BE) were used to trigger particular starch functionalities.

69 ## 2. Materials and methods

70 ### *2.1. Materials*

71 Corn starch was purchased from Miwon (Seoul, Korea). Amyloglucosidase (EC
72 3.2.1.3), fungal α-amylase (EC 3.2.1.1), cyclodextrin-glycosyltransferase (EC 2.4.1.19)
73 and branching enzyme (EC 2.4.1.18) activities were provided by commercial food grade
74 preparations (Amyloglucosidase 1100, Fungamyl 2500 SG, Toruzyme® 3.0 L and
75 Branchzyme) supplied by Novozymes (Bagsværd, Denmark). AMG activity was 1100

AGU/g (amyloglucosidase activity defined as the amount of enzyme that cleaves 1 μmol of maltose per min at 37 ºC); AM activity was 2500 FAU/g (fungal amylase activity); CGTase activity was 3 KNU/mL (kilo novo alpha amylase unit); BE activity was 50000 BEU/mL (branching enzyme units). All the other chemicals were analytical reagent grade and used without further purification. All solutions and standards were prepared by using deionized water.

2.2. Preparation of porous starch

The preparation of porous starch was based on the method of Dura *et al*. (2014; 2016) with minor modifications. Corn starch (20 g) was suspended in 100 mL of 20 mM sodium acetate buffer at pH 4.0 (AMG) or sodium phosphate buffer at pH 6.0 (AM, CGTase, BE). Then, different enzyme concentrations, expressed in units of enzyme stock solutions per grams of starch (U/g starch), were added to the starch suspensions, separately. The lowest enzyme concentration was the minimum recommended by the manufacturer (5.5 AMG U/g, 5.5 AM U/g, 0.1 CGTase U/g and 500 BE U/g), increasing concentrations (2, 3, 6 and 10 times the initial level) were also tested. Samples were kept in a shaking water bath (50 rpm) at 50 ºC for 2 h. Then samples were centrifuged for 15 min at 7000 ×*g* at 4 ºC. Supernatants were boiled in a water bath for 10 min to inactivate the enzymes before any further analyses. Sediments were washed twice with 50 mL of water, homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, USA) for 1 min at speed 3, and then centrifuged at the same conditions as before. Washed sediments were freeze-dried and kept at 4 ºC for subsequent analyses. Starch samples were subjected to the same procedure, without adding enzyme, at pH 4.0 (A-0) and pH 6.0 (P-0), and used as references. Two batches were prepared for each treatment.

2.3. Scanning electron microscopy (SEM)

The granule morphology of native, controls and treated starches was observed using a JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Samples were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan) prior to observation. The obtained samples were examined at an accelerating voltage of 10 kV and magnified 3,500x times.

The microstructure analysis was carried out using the image analysis program (ImageJ, UTHSCSA Image Tool software). The SEM images were saved as 8-bit tiff format. Scale was initially set using the relationship between pixels and known distance. Threshold was assessed applying the default algorithm and then particle analysis was carried out. The following parameters were measured: granule size and the pore size. The area occupied by pores in a starch granule was calculated as the sum of the areas of all the pores of a starch granule divided by granule pore. Values were the average of 20 independent measurements.

2.4. High performance anion exchange chromatography (HPAEC)

The hydrolysis compounds (oligosaccharides and CDs) lixiviated during enzymatic treatment were quantified according to Dura and Rosell (2016). Samples were filtered through a 0.45 μm pore size membrane (Millex-HV) and then injected (10 μL) into HPAEC through a CarboPac PA-100 column (250 mm × 4 mm) at flow rate 1.0 mL/min, coupled to a pulsed amperometric detector (Dionex). Solutions included: A (water), B (1 mol/L NaOH) and C (1 mol/L C2H3NaO2). Running profile applied was: time zero, 92.5% A, 5% B, 2.5% C; 25 min, 85% A, 5% B, 10% C; 1 min, 70% A, 15% B, 15% C; 3 min, 66% A, 15% B, 19% C; 5 min, 57% A, 15% B, 28% C; 1.5 min, 37% A, 15% B, 48% C. Standards of known concentrations were previously analyzed.

2.5. Amylose content of enzymatically treated starches

The amount of amylose of the starches was analyzed in triplicate using a commercial assay kit (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) based on the concanavalin A method (Gibson, Solah & McCleary, 1997).

2.6. Damage starch

Damaged starch levels were estimated at least in duplicate following the American Association of Cereal Chemists, method 76-31.01 (2000).

2.7. Adsorption of water and sunflower oil

Adsorptive capacity of starches for water and sunflower oil were determined according to the method described by Yousif, Gadallah and Sorour (2012) with a slight modification. Starch (0.1 g) and solvent (1 mL, water or oil) were mixed and vortexed for 30 min at room temperature. Slurries were centrifuged 10 minutes at 3,000 x *g* and decanted. When no more water or sunflower oil was dropped off onto the filter paper, weight of the sediment was measured. The adsorption capacity was calculated as the weight of the wetted sediment divided by the dry weight of sample (g/g).

2.8. Viscosity measurement

The pasting properties of native and enzymatically modified starches were measured with the Rapid Visco Analyzer (RVA-4500, Perten Instruments, Hägersten, Sweden). Starch (2 g based on 14% moisture content) was added to 20 mL of water placed into the aluminum RVA canister. Slurries underwent a controlled heating and cooling cycle, from 50 to 95 ºC in 282 s, holding at 95 ºC for 150 s and then cooling to 50 ºC. The initial speed for mixing was 960 rpm for 10 s, followed by a 160 rpm paddle speed that was maintained for the rest of assay. Pasting parameters such as pasting temperature, peak viscosity, breakdown (peak viscosity-hot paste viscosity), final viscosity, setback (cold paste viscosity-peak viscosity) were recorded using Thermocline software for Windows (Perten Instruments, Hägersten, Sweden).

2.9. DSC thermal analysis

The gelatinization characteristics of modified starches were determined using a differential scanning calorimetry (DSC) from Perkin–Elmer (DSC 7, Perkin–Elmer Instruments, Norwalk,CT). The slurry of starch and water (3:1) was placed into stainless steel capsules. Capsules were hermetically sealed and equilibrated at room temperature for one hour before analysis. The samples were scanned from 30 to 120 °C at a heating rate of 10 °C/min under nitrogen atmosphere, using an empty stainless steel capsule as reference. The temperature values obtained were the onset temperature (To), peak temperature (Tp), and conclusion temperature (Tc). The enthalpy of gelatinization (ΔH) was estimated based on the area of the main endothermic peak, expressed as joule per gram sample (J/g).

2.10. Statistical analysis

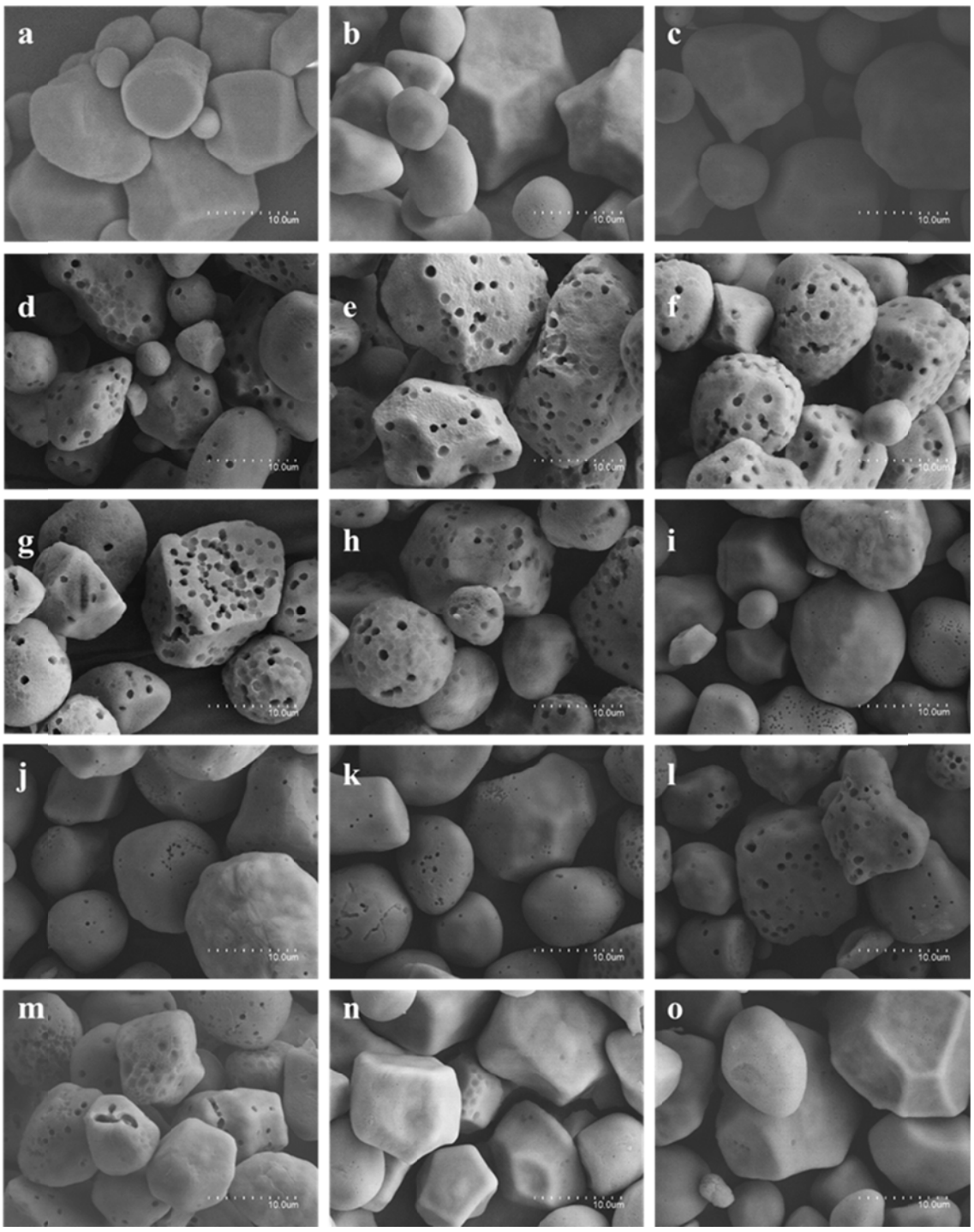
All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean ± standard deviation. Fisher's least significant differences test was used for assessment of significant differences among experimental mean values with 95% confidence. Statistical computations and analyses were conducted using Statgraphics Centurion XV software (Bitstream, Cambridge, N).

3. Results

3.1. Microstructure analysis

The shape, size, structure and surface characteristics of corn starch granules tested (native, references and treated starches) were investigated using SEM (Figure 1). Native starch granules displayed an irregular and mostly polygonal shape with relatively smooth surface (Figure 1a). Reference starches (Figure 1 b,c) had similar appearance to native starch, showing no evidence of rupture, breakage or pores due to the incubation

179 with buffer; results that were analogous to those reported previously (Dura, Błaszczak
180 & Rosell, 2014; Dura & Rosell, 2016). The effect of enzymatic treatment was readily
181 visible in the modified starches microstructure, obtaining in all cases porous starch
182 granules, without affecting the shape of the granule (Figure 1 d-w).



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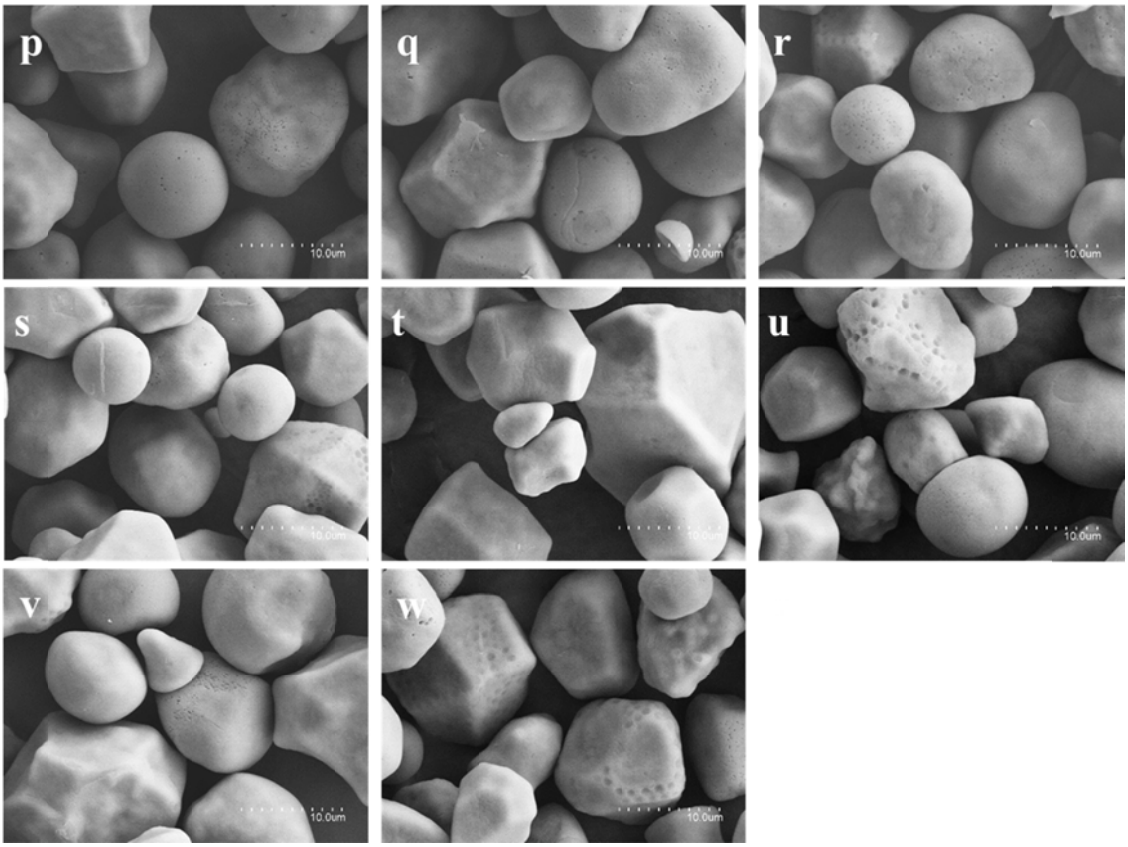

Figure 1: Scanning electron micrograph of and native corn starch (a), samples treated enzymatically (d-w) and their counterparts controls (b and c). Magnification 3500×. Reference A-0 (b); Reference P-0 (c); AMG 5.5, 11, 16.5, 33 and 55 (d-h); AM 5.5, 11, 16.5, 33 and 55 (i-m); CGTase 0.1, 0.2, 0.3, 0.6 and 1 (n-r); BE 500, 1000, 1500, 3000 and 5000 (s-w). Numbers following enzyme abbreviations are referred to the enzyme activity applied.

To give some objective results about the action of the enzymes, the pore size and the ratio pore area to starch granule area (related to the abundance of pore per granule) were quantified using image analysis (Figure 2). The pore size as well as pore area distribution was significantly affected by the type of enzyme and also their level. AMG action resulted in starch granules with larger pores and wider size distribution (Figure 2 A). In opposition, CGTase led to the lowest pore size. As the concentration of AMG increased, the size of the pores progressively augmented until 16.5 U of AMG were added; at higher enzyme level no further pore size increase was observed, although a significant increase in the ratio pore area to granule area was observed (Figure 2 B) indicating more pores per granule. Nevertheless, it was noted that at higher AMG concentrations appeared some depressions in the granules, which resulted from the

eroding action of the enzyme onto the granule surface. Aggarwal and Dollimore (2000) also observed a visible increase in the size of the pores when augmented the AMG concentrations, till enzyme activity (800 U/g starch) was so pronounced that walls around pinholes were broken, leading to large irregular holes and broken structure. Similarly, pore size increased with the amount of AM or CGTase added, although both treatments resulted in smaller pore size than AMG treatment. The ratio pore to granule area of AM treated starches also maintained a similar pattern to the AMG samples, while it remained constant when CGTase enzyme was used. The BE enzyme produced very irregular pore sizes without any trend with the level of enzyme. It should be noted that the pore size was bigger when lower concentrations of enzymes were used, but in those cases pores resembled wide craters instead of deep holes. At higher enzyme concentration, smaller and deeper pinholes appeared, leading a mixture of heterogeneous sizes.

When starch granules are incubated with amylolytic enzymes, the enzymes migrate through the channels and initiate hydrolysis leading to an inside out pattern of digestion (Chen & Zhang, 2012). Nevertheless, the present study reveals that different porous starches could be obtained depending on the type, thus it is possible to modulate the number and size of pores by using either different amylolytic enzyme or level of enzyme.

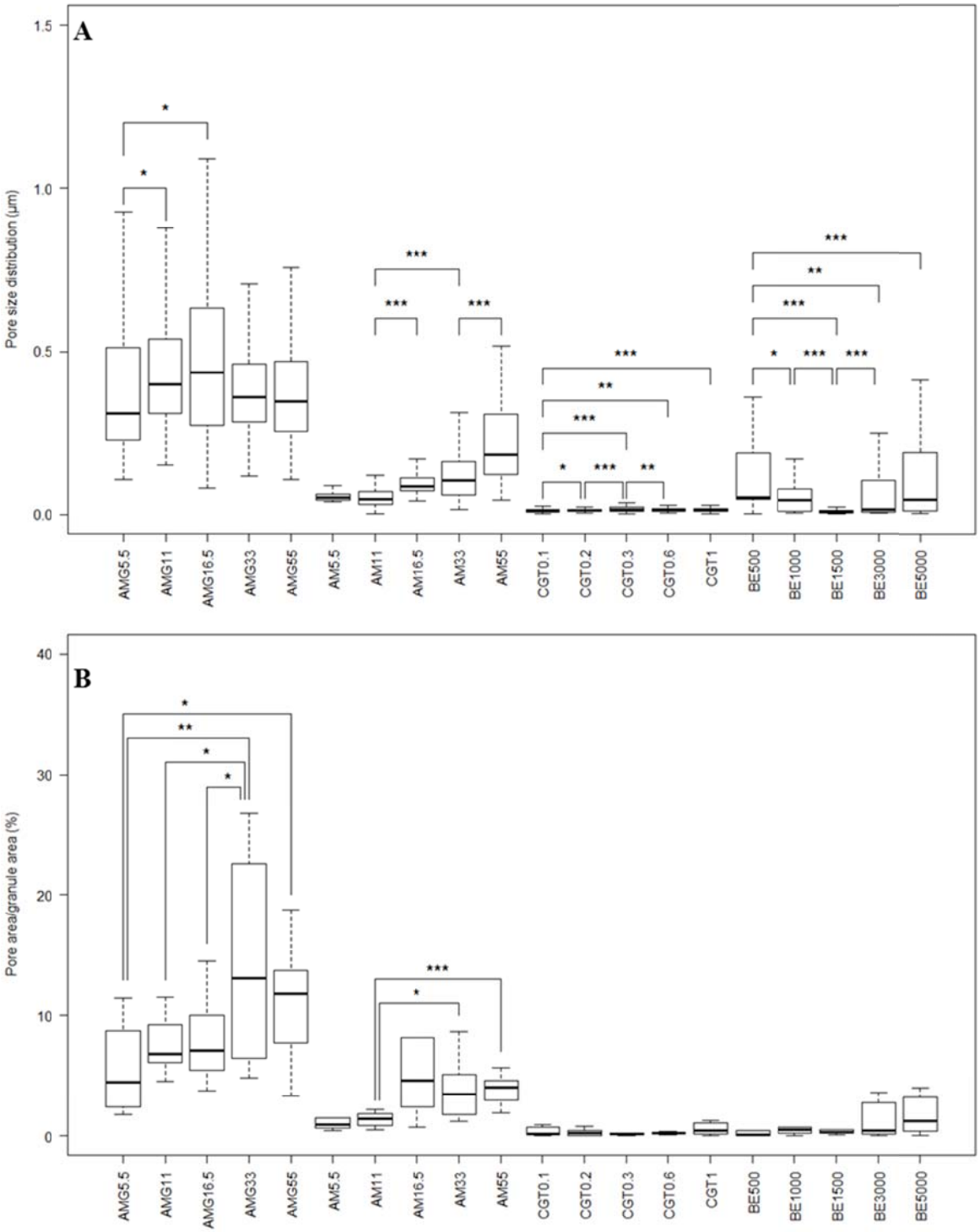

Figure 2: Image analysis from SEM photographs. A) Pore size and B) pore surface area distribution for each enzyme by boxplot. Numbers following enzyme abbreviations are referred to the enzyme activity applied.

3.2. CDs and oligosaccharides released during enzymatic treatment

To understand the action of the enzymes on the starch granules, the released compounds after the incubation were analyzed. Table 1 listed the oligosaccharides and cyclodextrins contents released per starch (mg 100 g^{-1}). As expected, neither oligosaccharides nor cyclodextrins (CDs) were released from the reference samples (data not shown), neither from AMG treatment. No oligosaccharides (from DP1 to DP5) were released when corn starches were subjected to BE hydrolysis. BE cleaves α-(1 → 4)-O-glycosidic bonds and transfers the cleaved-glucan to α-(1 → 6) position leading to branched glucan mixtures (Roussel et al., 2013).

Table 1: Oligosaccharides and cyclodextrins released after corn starch hydrolysis by AMG, AM and CGTase. Results are expressed in mg 100 g^{-1} of starch.

Enzyme type	Enzyme (U/g starch)	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentaose	α-CD	β-CD
AMG	5.5	16.19 ± 1.31	n.d	n.d	n.d	n.d	n.d	n.d
	11	15.64 ± 1.39	n.d	n.d	n.d	n.d	n.d	n.d
	16.5	16.16 ± 1.17	n.d	n.d	n.d	n.d	n.d	n.d
	33	15.57 ± 1.08	n.d	n.d	n.d	n.d	n.d	n.d
	55	15.49 ± 1.01	n.d	n.d	n.d	n.d	n.d	n.d
AM	5.5	9.76 ± 0.04	10.81 ± 0.20	7.68 ± 0.13	2.05 ± 0.02	n.d	n.d	n.d
	11	11.60 ± 0.27	8.82 ± 0.22	3.23 ± 0.40	1.90 ±0.14	0.18 ± 0.00	n.d	n.d
	16.5	12.42 ± 0.06	9.48 ± 0.39	2.38 ± 0.17	1.57 ± 0.38	n.d	n.d	n.d
	33	13.94 ± 0.41	9.70 ± 0.13	0.55 ±0.05	1.18 ± 0.01	n.d	n.d	n.d
	55	15.23 ±0.16	10.49 ± 0.20	0.27 ± 0.09	0.42 ± 0.08	n.d	n.d	n.d
CGTase	0.1	1.23 ± 0.03	0.54 ± 0.05	0.51 ± 0.09	0.50 ± 0.13	0.01 ± 0.00	2.25 ± 0.09	n.d
	0.2	1.37 ±0.03	1.07 ± 0.00	0.85 ± 0.04	0.96 ± 0.06	0.02 ± 0.00	2.33 ± 0.06	n.d
	0.3	0.83 ±0.04	1.07 ± 0.05	0.93 ± 0.04	1.22 ± 0.09	0.02 ± 0.00	2.73 ± 0.24	n.d
	0.6	0.70 ±0.08	1.19 ± 0.17	0.97 ± 0.13	1.00 ± 0.13	0.01 ± 0.00	1.73 ± 0.02	n.d
	1	1.27 ± 0.02	1.78 ± 0.00	1.37 ± 0.02	1.60 ± 0.05	0.03 ± 0.00	2.09 ± 0.14	n.d

n.d. non detected

233 Regarding the other amylolytic enzymes, starch-converting enzymes have been
234 classified into exo-amylases and endo-amylases owing to their cleavage action, and
235 results displayed that difference (Table 1). AMG treatment released exclusively glucose,
236 and the amount remained constant independently on the enzyme concentration.
237 Amyloglucosidase is a well-known exo-amylase, releasing only glucose residues from
238 amylose or amylopectin chains (Bouchet-Spinelli, Coche-Guérente, Armand, Lenouvel,
239 Labbé & Fort, 2013). However, saturation of the non-reducing-ends of starch chains has
240 been reported when enough glucoamylase is present (Chen & Zhang, 2012), which
241 would explain the steady glucose level.
242 In addition, the endo-amylases, AM and CGTase, are able to cleave α-1–4 glycosidic
243 bonds existing in the internal part (endo-) of a polysaccharide chain. As expected, AM
244 majorly converted starch to glucose followed by maltose. Moreover, the amount of
245 short chain oligosaccharides, ranging from DP1 to DP2 increased with the amount of
246 AM added, whereas DP3, DP4 and α-CD chains decreased. Conversely, the amount of
247 short chain oligosaccharides ranging from DP1 to DP5 decreased as increasing the level
248 of CGTase added, with a simultaneous increase in α-CD. Overall, CGTases convert
249 amylose or amylopectin into a mixture of α-, β- and γ-CD and some dextrins, and the
250 proportion was dependent on the enzyme specificity (Terada, Yanase, Takata, Takaha &
251 Okada, 1997), but also on the substrate, complexing agents and reaction conditions
252 (Blackwood & Bucke, 2000).
253 *3.3. Amylose, damaged starch content and adsorptive capacity*
254 Amylose and damaged starch contents were determined in the treated starches (Table
255 2). The statistical analysis indicated that the enzymatic treatment significantly modified
256 the amylose content, the amount of damage starch and the adsorption properties of the
257 starches; but the enzyme level only prompted significant effect on the amount of

damage starch and adsorptive water capacity. Amylose content showed a significant moderate correlation with the damaged starch content (r=0.6684, P<0.0000), mainly ascribed to the action of AMG and BE. Concerning the specific action of each enzyme, a significant reduction in amylose content, with the subsequent increase in amylopectin, was observed after AM and CGTase treatments, without observing any trend with the level of enzyme applied. These results are in agreement with the inverse relationship reported between the amylose content and the amount of hydrolyzed starch (Tester, Qi & Karkalas, 2006), and also with the trend reported for CGTase modified starches (Dura & Rosell, 2016),. Nevertheless, previous results with AM and AMG indicated that at lower concentrations than the one of the present study no change in the amylose content was observed even when increasing the enzymatic treatment to 24 or 48 hours (Dura, Błaszczak & Rosell, 2014).

Damaged starch was hardly affected by the action of AM and CGTase, although a tendency to decrease it was observed in the case of CGTase. Considering that microstructure analysis confirmed the impairment of the granule, it seems that the experimental assay for quantifying damage starch was not sensible or reliable enough to distinguish the degree of damage. Conversely, AMG and BE treatment promoted the opposite trend, the amylose content appeared to increase but not always significantly, and the amount of damage starch significantly augmented, particularly in the case of BE. Regarding the level of BE applied, a clear decrease of damage starch content was observed when increasing the enzyme concentration. Starch granules have a unique semi-crystalline supramolecular structure with concentric layers of amorphous and crystalline regions radiating from the hilum (Ratnayake & Jackson, 2008). Taking into account that the amylopectin side chains form the framework of the crystalline lamellae, with branching points located in the amorphous domains, where the majority of the

283 amylose is located (Copeland, Blazek, Salman & Tang, 2009), it seems that depending
284 on the enzymatic treatment amylose or amylopectin are preferentially hydrolyzed.
285 Results on amylose content suggested that AM and CGTase attacked more proportion
286 of amylose, leading an increase in the amount of amylopectin, suggesting deeper
287 pinholes and the attack of amorphous and crystalline structure. In opposition, AMG and
288 BE seem to hydrolyze preferentially the amylopectin chains, increasing the proportion
289 of amylose in the surface of starch granule, thus bigger and less deep holes, which
290 agrees with microstructure results.

291 Table 2: Effect of enzymatic treatment on the water and oil adsorption capacity and chemical composition (amylose content and damaged starch) of the

292 resulting porous starches

Enzyme type	Enzyme (U/g starch)	Amylose content (%)	Damaged starch (%)	Adsorptive water capacity (g/g)	Adsorptive oil capacity (g/g)
Native	0	25.76 ± 0.82 ^{de}	15.41 ± 0.19 ^{cd}	0.74 ± 0.02 ^{a}	1.14 ± 0.05 $^{g-h}$
AMG	5.5	23.47 ± 0.35 ^{cd}	21.30 ± 0.05 ^{e}	1.12 ± 0.03 ^{hi}	1.10 ± 0.05 $^{e-h}$
	11	27.36 ± 1.31 $^{e-g}$	22.77 ± 0.17 ^{f}	1.25 ± 0.04 ^{j}	1.27 ± 0.00 $^{h-j}$
	16.5	26.97 ± 0.31 $^{e-g}$	23.64 ± 0.15 ^{f}	1.45 ± 0.08 ^{k}	1.41 ± 0.02 ^{j}
	33	28.01 ± 4.76 $^{e-g}$	21.51 ± 0.07 ^{e}	1.44 ± 0.08 ^{k}	1.35 ± 0.02 ^{j}
	55	26.91 ± 0.16 ^{g}	20.66 ± 0.05 ^{e}	1.46 ± 0.06 ^{k}	1.32 ± 0.03 ^{ij}
AM	5.5	19.53 ± 1.82 ^{ab}	14.97 ± 0.05 $^{a-d}$	1.16 ± 0.06 ^{ij}	0.85 ± 0.28 $^{a-d}$
	11	18.56 ± 0.46 ^{ab}	15.01 ± 0.63 $^{a-d}$	1.07 ± 0.01 $^{g-i}$	0.96 ± 0.08 $^{c-f}$
	16.5	18.95 ± 0.38 ^{ab}	15.40 ± 0.22 ^{cd}	0.85 ± 0.03 $^{b-e}$	0.76 ± 0.08 $^{a-c}$
	33	21.24 ± 0.41 $^{a-c}$	15.13 ± 0.37 $^{b-d}$	0.71 ± 0.06 ^{a}	0.86 ± 0.08 $^{a-d}$
	55	19.17 ± 0.82 ^{ab}	16.03 ± 0.73 ^{d}	0.93 ± 0.03 $^{d-f}$	0.71 ± 0.01 ^{ab}
CGTase	0.1	21.26 ± 0.19 $^{a-c}$	14.38 ± 0.05 $^{a-c}$	0.90 ± 0.07 $^{c-f}$	0.86 ± 0.05 $^{a-d}$
	0.2	19.45 ± 1.07 ^{ab}	14.37 ± 0.19 $^{a-c}$	0.89 ± 0.04 $^{f-h}$	1.09 ± 0.10 $^{e-h}$
	0.3	19.58 ± 2.39 ^{ab}	13.68 ± 0.07 ^{a}	0.97 ± 0.07 $^{c-f}$	0.98 ± 0.17 $^{d-g}$
	0.6	21.91 ± 0.14 ^{bc}	13.05 ± 0.91 $^{a-b}$	0.80 ± 0.03 $^{e-g}$	1.13 ± 0.33 $^{f-i}$
	1	21.66 ± 0.64 ^{bc}	14.41 ± 0.10 $^{a-c}$	0.93 ± 0.04 $^{a-c}$	0.96 ± 0.27 $^{c-g}$
BE	500	28.96 ± 0.15 ^{fg}	30.66 ± 0.11 ^{ij}	0.75 ± 0.07 ^{ab}	0.85 ± 0.01 $^{a-d}$
	1000	18.90 ± 0.84 $^{d-f}$	31.18 ± 0.63 ^{j}	0.79 ± 0.04 $^{a-c}$	0.66 ± 0.09 ^{a}

		1500	28.54	±	1.36	e-g	29.61	±	1.39	hi	0.88	±	0.02	c-f	0.90	±	0.00	b-e
		3000	27.11	±	1.65	d-f	29.06	±	1.22	h	0.82	±	0.05	a-d	0.85	±	0.11	a-d
		5000	27.25	±	0.65	e-g	27.76	±	2.06	g	0.87	±	0.12	b-f	0.84	±	0.12	a-d
P-value	**Enzyme type**		0.00				0.00				0.00				0.00			
	Enzyme (U/g)		0.11				0.00				0.02				0.17			

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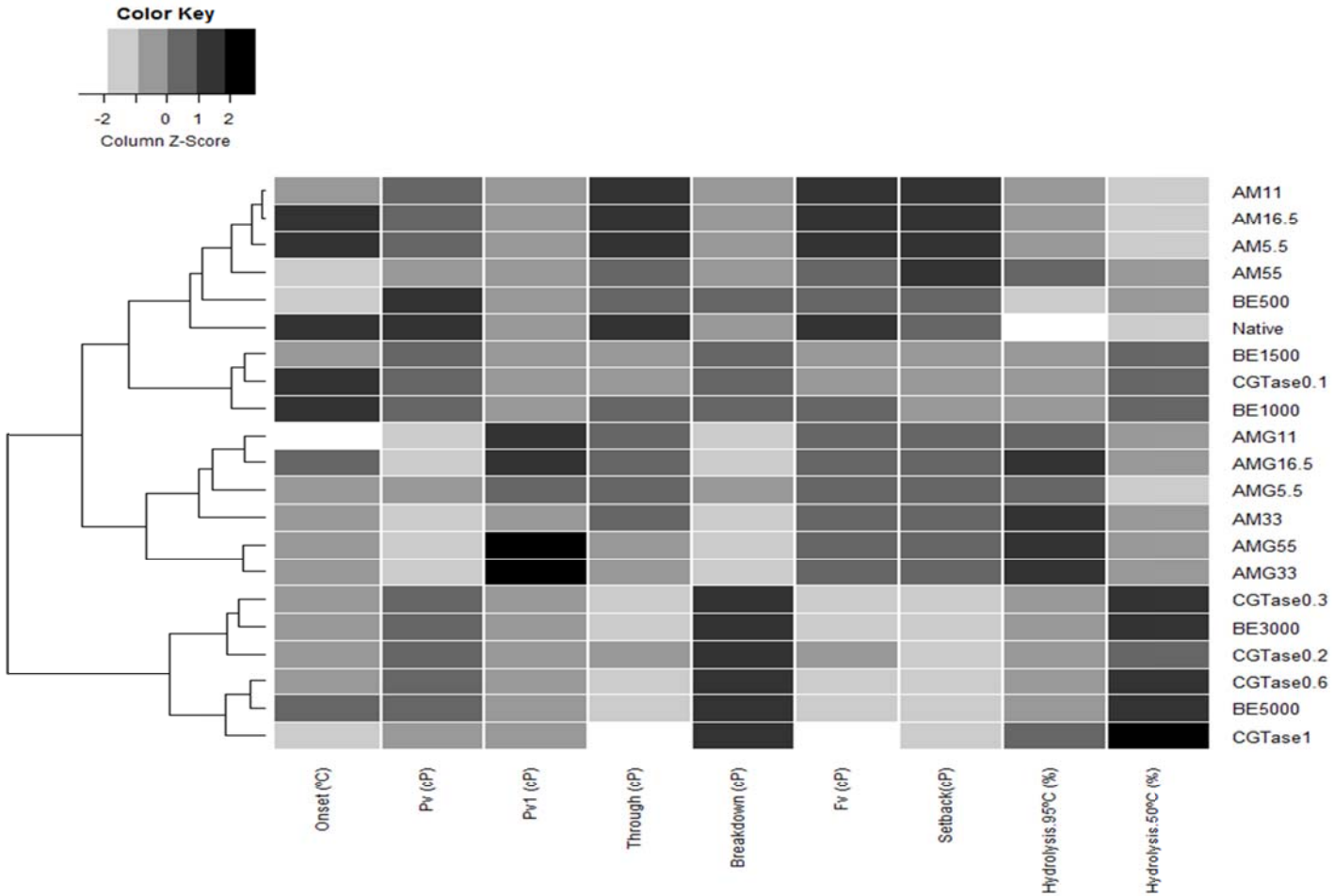
The adsorptive capacity of modified starches for water and sunflower oil are also summarized in Table 2. The hydrophilic nature was significantly dependent on both enzyme type and concentration, while hydrophobic nature depended only on the enzyme type. In general, all enzymatic treatments increased the water adsorption capacity of the starches; among them, AMG showed the greatest effect, followed by AM, CGTase and BE treatment. Likely, the size of the pores originated by AMG was responsible of this behavior due to the increase of the surface area. The adsorptive oil capacity of starch was only significantly modified when treated with AMG. Chen and Zhang (2012) obtained an increase in both solvents retention ability respect to native starch, due to the increase in the surface area promoted by the starch treatment with AMG (11 U/g starch), which agrees with results of the present study. Therefore, it seems that the pore size plays a fundamental role for oil adsorption, which was only sufficient in the case of AMG hydrolysis.

3.4. Enzymatic modification effects on pasting and thermal starch properties

To illustrate the pasting characteristics of the porous starches obtained from different type of enzymes a heatmap was constructed with the pasting properties (Figure 3). The heatmap of the hierarchical clustering of the RVA properties for the modified samples was analyzed on the basis of similarities and differences in starch pasting properties, including onset, peak viscosity, through, breakdown, final viscosity, setback, hydrolysis percentage at 95 °C and 50 °C (Figure 3). The dendrogram consisted of three major clusters. One cluster contained native, AM treated samples and the minor concentration of CGTase and BE treatments, up to 1500 U/g starch. Another cluster essentially included AMG treated starches and one AM treated sample. The last cluster comprised CGTase and BE treated starches using high enzyme levels.



318

319 Figure 3: Hierarchical clustering of RVA profiles. A heat map representing the hierarchical clustering of the Z scores of the enzyme activities related to viscoelastic
320 properties, when compared AMG, AM, CGTase and BE enzyme treatment. The Z scores represent the dispersion around the overall mean of the viscoelastic properties and
321 weighted by their standard errors. The scale of the intensity is shown in the top corner. Rows represent samples and column viscoelastic properties. Numbers following
322 enzyme abbreviations are referred to the enzyme activity applied. Pv: peak viscosity; Pv1: additional peak viscosity; Fv: final viscosity

323 It was evident from the heatmap that enzymes changed the pasting performance of
324 starch suspensions and the effect was also dependent on their concentrations,
325 particularly in the case of CGTase and BE. The onset temperature, indicative of the
326 initial viscosity increase, was significantly decreased by all enzyme studied,
327 independently of the concentration used. Therefore, lower cooking temperature was
328 required for the gelatinization of porous starches, likely due to faster water absorption
329 on the starch granules, since a negative correlation was observed between onset
330 temperature and pore size (r = -0.4581, $P < 0.001$). AM treated samples showed similar
331 pasting behavior to native starch, unless the maximum viscosity that decreased after
332 treatment. AM acts on the starch molecules breaking α-(1-4) linkages and providing
333 dextrins, which present lower swelling during gelatinization (Rocha, Carneiro &
334 Franco, 2010). Porous starches had significantly lower peak viscosity, through, final
335 viscosity and setback compared to native, which agree with previous results (Dura,
336 Błaszczak & Rosell, 2014). In the case of AMG treated samples they were grouped due
337 to their lower peak viscosity and breakdown and higher final viscosity and setback,
338 besides the presence of an additional peak viscosity (Pv1) during heating, prior to the
339 common peak viscosity at 95 ºC. This additional peak was negatively correlated with
340 peak viscosity, showing a progressive increase in the first peak in parallel to the
341 reduction of peak viscosity. The decrease of peak viscosity due to the joint action of α-
342 amylase and glucoamylase has been explained by the disintegration of fragile granules
343 owing to their porous structure, leading to less viscous slurries (Uthumporn, Zaidul &
344 Karim, 2010). In this regard, pore size, ratio of pore area to granule area and water
345 adsorptive capacity was negatively correlated with peak viscosity, confirming this
346 hypothesis.

347 Porous starches obtained with very high levels of CGTase or BE were mainly
348 characterized by very low values of final viscosity and setback, and high breakdown
349 values. Those effects have been reported when wheat starch was treated by CGTase
350 Gujral and Rosell (2004).
351 The values for the thermal properties of native starch (Table 3) agrees with previous
352 reported results for corn (Jane et al., 1999). In modified starches, To, Tp and ΔH
353 significantly ($P < 0.05$) varied owing to the type of enzyme used and its level, but Tc
354 was only significantly affected by the type of enzyme. Porous starches showed lower To
355 and Tc than native starch. In the case of AMG treated starches those temperatures
356 decreased when increasing the level of enzyme during treatment. Moreover, lower
357 energy (ΔH) was required to promote starch gelatinization, likely due to less energy was
358 needed to unravel and melt the unstable double helices during gelatinization (Chung,
359 Liu & Hoover, 2009).
360 On the other hand, BE enzyme produced starches with lower To and Tp, but similar Tc
361 to native starch. Conclusion temperature (Tc) was only significantly reduced by AM.
362 Correlation analysis indicated that all gelatinization parameters evaluated except
363 enthalpy were positively correlated ($P < 0.05$) with amylose content, but not with
364 damaged starch, pore size or pore area to starch granule, which are in agreement with
365 previous observations (Stevenson, Doorenbos, Jane & Inglett, 2006). In addition,
366 enthalpy was negatively correlated with water ($r = -0.3555$, $P < 0.05$) and oil adsorption
367 capacity ($r = -0.4078$, $P < 0.01$).
368

369 Table 3: Thermal properties of enzymatically modified corn starches determined by DSC

Enzyme type	Enzyme (U/g starch)	T_0 (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)
Native	0	63.28 ± 0.14 ^{i}	68.20 ± 0.00 ^{h}	74.71 ± 0.17 ^{b}	20.66 ± 1.27 $^{c-e}$
AMG	5.5	62.96 ± 0.21 $^{g-i}$	66.70 ± 0.24 $^{a-e}$	74.32 ± 0.68 ^{b}	20.26 ± 1.08 $^{b-e}$
	11	63.26 ± 0.10 ^{hi}	67.53 ± 0.00 ^{g}	74.86 ± 0.08 ^{b}	19.18 ± 1.70 ^{bc}
	16.5	63.26 ± 0.15 ^{hi}	67.37 ± 0.47 ^{fg}	74.65 ± 0.11 ^{b}	16.64 ± 0.14 ^{aa}
	33	62.80 ± 0.57 $^{f-h}$	67.03 ± 0.71 $^{c-g}$	74.45 ± 0.92 ^{b}	19.64 ± 1.75 $^{b-d}$
	55	62.65 ± 0.47 $^{d-g}$	66.95 ± 1.06 $^{b-g}$	73.88 ± 1.43 ^{b}	19.06 ± 0.38 ^{bc}
AM	5.5	62.00 ± 0.36 $^{a-c}$	66.45 ± 0.12 $^{a-c}$	73.93 ± 0.04 ^{a}	20.77 ± 0.18 $^{c-e}$
	11	61.86 ± 0.50 ^{a}	66.28 ± 0.35 ^{a}	73.81 ± 0.62 ^{a}	23.37 ± 1.13 ^{f}
	16.5	61.93 ± 0.20 ^{a}	66.37 ± 0.00 ^{ab}	73.86 ± 0.06 ^{a}	19.43 ± 0.49 $^{b-d}$
	33	62.24 ± 0.22 $^{a-e}$	66.70 ± 0.24 $^{a-e}$	73.12 ± 0.40 ^{a}	19.82 ± 2.70 $^{b-e}$
	55	61.98 ± 0.11 ^{ab}	66.37 ± 0.24 ^{ab}	73.62 ± 0.13 ^{a}	21.67 ± 0.94 $^{d-f}$
CGTase	0.1	62.49 ± 0.12 $^{c-g}$	67.28 ± 0.12 $^{e-g}$	73.98 ± 0.12 ^{ab}	19.35 ± 1.39 ^{bc}
	0.2	61.99 ± 0.01 $^{a-c}$	66.37 ± 0.24 ^{ab}	73.27 ± 0.46 ^{ab}	20.99 ± 0.87 $^{c-e}$
	0.3	62.01 ± 0.12 $^{a-c}$	66.37 ± 0.00 ^{ab}	73.34 ± 0.18 ^{ab}	18.15 ± 0.56 ^{ab}
	0.6	62.20 ± 0.08 $^{a-d}$	66.62 ± 0.12 $^{a-d}$	73.68 ± 0.09 ^{ab}	19.47 ± 1.02 $^{b-d}$
	1	62.46 ± 0.10 $^{b-g}$	67.03 ± 0.24 $^{c-g}$	73.67 ± 0.26 ^{ab}	19.11 ± 0.58 ^{bc}
BE	500	62.81 ± 0.28 $^{f-h}$	67.28 ± 0.12 $^{e-g}$	74.25 ± 0.46 ^{ab}	23.72 ± 1.00 ^{f}
	1000	62.73 ± 0.40 $^{e-g}$	67.03 ± 0.24 $^{c-g}$	74.18 ± 0.96 ^{ab}	21.95 ± 1.43 ^{ef}
	1500	62.30 ± 0.05 $^{a-f}$	66.78 ± 0.12 $^{a-f}$	73.30 ± 0.24 ^{ab}	20.31 ± 0.84 $^{b-e}$
	3000	62.48 ± 0.28 $^{b-g}$	67.12 ± 0.35 $^{d-g}$	74.04 ± 0.77 ^{ab}	20.94 ± 1.39 $^{c-e}$
	5000	62.47 ± 0.32 $^{b-g}$	66.87 ± 0.24 $^{a-f}$	73.76 ± 0.19 ^{ab}	20.02 ± 0.70 $^{b-e}$
P-value	**Enzyme type**	**0.00**	**0.01**	**0.04**	**0.03**
	Enzyme (U/g)	**0.00**	**0.00**	**0.06**	**0.03**

370

To = onset temperature, Tp = peak temperature, Tc = conclusion temperature, ΔH = enthalpy change. Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 3).

4. Conclusions

Porous starches could be obtained by enzymatic treatment of corn starch at sub-gelatinization temperature. The size distribution of the pores and their area were dependent on the type of enzyme used for the starch treatment, but also the level of enzyme. AMG led to porous starches with larger holes, whereas the smallest were obtained with CGTase. Porous starches differed in their pasting performance and thermal properties, besides adsorptive water or oil capacities. By selecting the type of enzyme and its level it could be modulated the degree of porosity.

Enzymatic treatment of native starch granules reveals as a powerful tool to modify the properties of starch. The added value and feasibility of this methodology on different sources of starch should be examined.

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