Changes in physicochemical properties and *in vitro* starch digestion of native and extruded maize flours subjected to branching enzyme and maltogenic α-amylase treatment

Laura Román*[1], Mario M. Martínez[1, 2], Cristina M. Rosell[3], Manuel Gómez[1]
[1]Food Technology Area. College of Agricultural Engineering, University of Valladolid, 34004 Palencia, Spain
[2]Currently at: Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, 745 Agriculture Mall Drive, West Lafayette, IN 47907, USA.
[3]Institute of Agrochemistry and Food Technology (IATA-CSIC). Avda Agustin Escardino, 7. Paterna-46980, Spain.

*Corresponding author e-mail: laura.roman@iaf.uva.es

Abstract
Extrusion is an increasingly used type of processing which combined with enzymatic action could open extended possibilities for obtaining clean label modified flours. In this study, native and extruded maize flours were modified using branching enzyme (B) and a combination of branching enzyme and maltogenic α-amylase (BMA) in order to modulate their hydrolysis properties. The microstructure, pasting properties, *in vitro* starch hydrolysis and resistant starch content of the flours were investigated. Whereas BMA treatment led to greater number of holes on the granule surface in native samples, B and BMA extruded samples showed rougher surfaces with cavities. A reduction in the retrogradation trend was observed for B and BMA native flours, in opposition to the flat pasting profile of their extruded counterparts. The glucose release increased gradually for native flours as the time of reaction did, whereas for extruded flours a fast increase of glucose release was observed during the first minutes of reaction, and kept till the end, indicating a greater accessibility to their porous structure. After 16 hours of hydrolysis, resistant starch content was lower for treated extruded samples. These results suggested that, in enzymatically treated extruded samples, changes produced at larger hierarchical levels in their starch structure could have masked a slowdown in the starch digestion properties.
Keywords: Extrusion; branching enzyme; α-amylase; microstructure; pasting properties; starch hydrolysis

1 Introduction

Extrusion process is a hydrothermal treatment in which starchy products are subjected to high temperatures and mechanical shearing at short times and relatively low levels of moisture content [1]. Besides starch gelatinization, this treatment can also promote the breakage of the amylose and amylopectin chains (dextrinization), denaturation of proteins, enzyme (in)activation and Maillard reactions [2]. The extent of these changes is dependent on the severity of the extrusion and give rise to different physicochemical characteristics of the resultant product [1,3,4].

Extrusion is widely used in snack and breakfast cereal productions, in which flour based products are extruded with the aim of obtaining the final product in one simple continuous process [5]. Meanwhile, flours and starches are also extruded to gelatinize, melt and fragment their starch so as to adapt its rheological and hydration properties to the emerging needs imposed by the new food trends [6]. However, during extrusion, the disruption of the starch granules (gelatinization) also makes starch more accessible and susceptible towards enzymatic hydrolysis leading to a more rapid conversion of starch into glucose [3,4], which make these extruded products being rapidly digested and absorbed by our digestive system. According to recent studies, the long-term consumption of fast digestible products may contribute to promote human diseases such as type II diabetes, cardiovascular disease and obesity [7].

While those diseases have become major public health concerns worldwide, new enzymatic treatments have emerged in order to obtain healthier carbohydrates through the applications of different enzymes and techniques. Among these, branching enzyme (B) and maltogenic α-amylases (MA) have been used to slow down the digestion of starches. Branching enzyme (EC 2.4.1.18) catalyzes hydrolytic and transfer reactions to form new α-1,6 linkages [8]. Meanwhile, maltogenic α-amylase (EC 3.2.1.133) hydrolyze α-1,4-glucosidic linkages of starch and its derivatives to maltose reducing the chain length of the polymer [9]. MA also exhibits high transglycosylation activity via formation of various glycosidic linkages such as α-1,6 producing branched oligosaccharides [10]. In this way, the combination of branching followed by posterior trimming through a starch-active exo-hydrolase, such as β-amylase or maltogenic α-amylase, has been attempted to change the amylopectin fine structure to create resistant structures that slow down the starch digestion properties in native and cooked matrices. These changes mainly comprise increasing the ratio of short chains (degree of polymerisation, DP<13) to long chains of amylopectin and the relative amount of α,1-6 linkages [8,9,11]. Additionally, the formation of prebiotic isomaltooligosacharides has been also reported [8,12].

It is important to consider that native starch is only partially accessible for the enzyme catalysis, being necessary to promote previously the damage or breakage of the starch granules [13]. Therefore, whereas those previous treatments were carried out on starches, research on flours subjected to extrusion process is scarce. This treatment can be influenced by the interactions between starch and other non-starch components giving rise to different physicochemical properties than those of starch.

Even though Martínez et al. [12] reported the formation of molecular and supramolecular resistant structures in extruded flours, the effect of those structures on

the functional properties of flours, including their *in vitro* hydrolysis, has never been studied. Then, the objective of the current study was to investigate the impact of treatment with branching enzyme or a combination of branching enzyme and maltogenic α-amylase on the functional properties of native and extruded flours. With that goal, the *in vitro* starch digestion of these flours, the microstructure, pasting properties and resistant starch content were assessed.

2 Materials and Methods

2.1 Materials

Native maize flours (11.95 g/100 g moisture; 7.6 g/100 g protein) were supplied by Molendum Ingredients (Zamora, Spain). Extrusion of maize flour was carried out by Molendum Ingredients in a single screw extruder Bühler Basf (Bühler S.A., Uzwil, Switzerland). The length to diameter ratio for the extruder was 20:1. The extrusion conditions were carried out in order to ensure starch gelatinization (not detected gelatinization endotherm). Maize flour was extruded at a maximum barrel temperature of 160 ºC with further water addition of 12%, a feed rate of 500 kg/h and a screw speed of 453 rpm. Extruded product (7.1 g/100 g protein) was dried by convection air up to 9.32% of moisture content and then milled with a compression roller till particle size was lower than 200 microns. The extruded flour resulted after milling had a mean particle size of 99.37 μm.

Branching enzyme EC 2.4.1.18 (Branchzyme®; declared activity: 5000 BEU/g product) and maltogenic α-amylase EC 3.2.1.133 (Amylase™ AG 300 L; declared activity: 10000 MANU/g product), both from Bacillus subtilis, were gently provided by Novozymes (Bagsvaerd, Denmark). Chemical reagents from Sigma–Aldrich (Madrid, Spain) were of analytical grade.

2.2 Methods

2.2.1 Enzymatic treatment of flours

Enzymatic treatment was carried out according to Martínez et al. [12]. Briefly, native or extruded maize flours (6.0 g) were suspended into 30 mL of sodium acetate buffer (0.01 M, pH 5.0). The incubation was carried out with 300 U of B/g flour (B treatment) and a combination of 300 U of B and 7 U of MA/g flour (BMA treatment). The incubation was performed at 55 ºC for 2.5 h in a water bath. Then, 60 mL of an ethanol solution (96%, w/w) was added prior homogenization with an UltraTurrax homogenizer IKA-T25 (IKA works, Wilmington, USA) during one min at 13,000 rpm. Subsequently, samples were centrifuged for 10 min at 4000rpm. Flours were washed again and centrifuged at the same conditions as before. Sediments were dried at 45ºC for 48 h and milled for 20 s in a Moulinex super junior S (Groupe Seb Iberica, S.A, Barcelona, Spain). Flours were stored in airtight plastic containers perfectly sealed at 4 °C for further analysis. All treatments were made in duplicate. Non-treated native and extruded samples as well as native and extruded samples treated in the absence of enzyme were used as references in order to assess possible effect of the incubation and subsequent treatment.

2.2.2 Environmental scanning electron microscopy (ESEM)

Flour photomicrographs were taken with a Quanta 200FEI (Hillsboro, Oregon, USA) ESEM. Photomicrographs were taken in beam deceleration mode (BDM) at a landing energy of 1.5 KeV in high vacuum mode with a backscattered electron detector (BSED).

2.2.3 Pasting properties

Pasting properties of flours were analyzed using the standard method AACC [14], (AACC 61-02.01) with a Rapid Visco Analyser (RVA-4) (Perten Instruments, Hägersten, Sweden) controlled by Thermocline software (Perten Instruments, Hägersten, Sweden) for Windows. All flours were run in duplicate.

2.2.4 *In vitro* starch hydrolysis

In vitro starch digestibility was measured following the method described by Dura et al. [15] with slight modifications. Briefly, flour sample (0.1 g) was incubated with porcine pancreatic α-amylase (0.2 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 μL were withdrawn during the incubation period and mixed with 200 μL 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 μL) and the supernatants were pooled together and kept at 4 °C for further glucose enzymatic release.

Supernatant (100 μL) was diluted with 850 μL of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 50 μL 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. Four replicates were carried out for each determination.

Experimental data were fitted to a first-order equation [16]:

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

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.

2.2.5 Resistant Starch content

Starch hydrolysis was measured using AACC method 32-40-01 [14], modified by Gularte and Rosell [17]. Flours (0.1 g) placed in 10 mL Pyrex tubes was suspended in 2 mL of ethanol (80% w/w) and incubated at 85 °C in a shaking water bath (50 rpm) for 5 min and then centrifuged (2000 × *g*, 10 min, at room temperature). The precipitate was washed with 2 mL of ethanol (80% w/w) and centrifuged again, and supernatants were pooled together. The pellet after free sugar extraction was incubated with porcine pancreatic α-amylase (0.2 U/mL) in a shaking water bath at 37 °C for 16 h. Ethanol (96% w/w) was added to stop the enzymatic reaction, and the suspension was

centrifuged (10000 x *g* for 10 min, 4 ºC). The pellet was washed again (ethanol 50% w/w) and centrifuged at the same conditions as before. To quantify digestible starch, the supernatant (100 μL) was diluted with 850 μL sodium acetate pH 4.5, incubated (50 °C for 30 min) with 50 μL amyloglucosidase and released glucose was assessed as described for *in vitro* starch hydrolysis.

Resistant starch after 16 h hydrolysis was solubilized with 2 mL of 2 M KOH using a Polytron Ultra-Turrax homogenizer IKA-T18 (IKA Works, Wilmington, NC, USA) during 1 min at speed 3. The homogenate was diluted with 8 mL 1.2 M sodium acetate pH 3.8 and incubated with 100 μL amyloglucosidase (3480 U/mL) at 50 °C for 30 min in a shaking water bath and then centrifuged (2000 x *g* for 10 min, 4ºC). The glucose content of the resistant starch was measured using a glucose oxidase–peroxidase kit as described before and expressed in mg/100 mg of the sample in dry basis. Four replicates were carried out for each determination.

2.2.6 Statistical analysis

The obtained data were subjected to a one-way analysis of variance (ANOVA) using the LSD Fisher test ($P<0.05$). All analyses were performed with Statgraphics Centurion XVI software (StatPoint Technologies Inc., Warrenton, USA).

3 Results and Discussion

Enzymatic modification of the native and extruded maize flours was carried out independently with branching enzyme (B) and a combination of branching enzyme and maltogenic α-amylase (BMA). The action of each enzyme was compared with their specific control, which was submitted to the same treatment in the absence of enzymes (no enzyme), to eliminate possible responses owing to water suspension and drying processes. Furthermore, native and extruded flours not submitted to any treatment (non-treated) were as well used as control.

3.1 Microstructure of the flours

The effect of the enzymatic treatment on native and extruded flours is depicted in Fig. 1A and 1B. At low magnification (Fig. 1A), photomicrographs of non-treated native sample (a) showed tight aggregates of starch granules embedded into the protein matrix with some loose granules, whereas when subjected to the incubation in the presence or the absence of enzyme (c, e, g), this fully packed structure was no longer visible. In these samples, the starch granules appeared completely loose with some proteins bonded to them, but they kept the typical spherical and polygonal shape of maize granules [18].

At high magnification (Fig. 1B), these loose granules presented a smooth surface in non-enzymatically treated sample (c), where no holes were visible, neither for the native flour (a). Meanwhile, the two enzymatically treated native flours (e, g) showed changes in their surface, with several holes presented on it (See arrows). Very perforated granules were obtained in the BMA treatment compared with the B one, suggesting a synergetic action between both enzymes or a main action of maltogenic α-amylase on native starch.

At low magnification (Fig. 1A), photomicrographs of extruded samples exhibited the typical structure of pregelatinized flours in which the starch structure was lost and manifested by irregularly shaped or sized and nearly amorphous agglomerates [18,19]. Nevertheless, some rounded swollen starch granules were still visible in this broken structure. Despite the presence of those remained granules, gelatinization was complete, as shown in the previous study carried out by Martínez et al. [12], where these flours did not present endothermic gelatinization peak in DSC measurements. Thus, the presence of those swollen starch granules may be explained by the fact that during gelling which follows gelatinization step, the leaching and disintegration of the starch granules is not entire. In this way, Shrestha et al. [20] also observed some small intact granules in extruded starches, indicating that not all granules are destroyed during extrusion cooking and some remain apparently intact, 'hidden' within the sheared starchy mass.

At this magnification, only a clear coarser particle size of non-incubated extruded flour was visible (b). Finer particle size of the treated flours could be a result of the incubation treatment in which the water may act softening the flour structures and leading to more breakable structures during remilling. Meanwhile, at high magnification (Fig. 1B), for BMA and B treatments (f, h) it was observed that the surface of the granules seemed rougher with cavities or channels on it (See circles), which could indicate the access points of the enzyme into the starch matrix. Furthermore, in the case of BMA treatment, certain structures appeared pasted to each other, joined by a gel-like structure, which may be explained by the effect of some free sugars release that acted as gluing material. This is in accordance with the higher maltose content of this flour, as demonstrated by Martínez et al. [12], resulting from the catalysis action of maltogenic α-amylase.

Overall, micrographs analysis suggested that, to a certain extent, enzymatic treatment affected the external structure of flours but still keeping the particle shape.

3.2 Pasting properties

Pasting profile of native and extruded flours is displayed in Fig. 2. Native treated flours (no enzyme, B and BMA) showed an increase in the peak viscosity when compared to the non-treated native flour and a shorter time for reaching peak viscosity was observed in the enzymatically treated flours. The different pasting performance might be explained by differences in starch accessibility or particle size [21]. In fact, as microscopy results showed, treated flours presented a disaggregated structure of loose granules, which could have fostered water absorption, reaching the peak viscosity earlier. This greater accessibility may have also contributed to the increase in the peak viscosity, since starch granules are not physically restricted by the protein bodies and can swell more. Whilst in non-treated native flour the tight aggregated structure of starch granules could have hampered water absorption. Furthermore, the higher peak viscosity observed in treated flours can also be attributed to modest molecular rearrangements produced in the starch granules when held in an excess of water at temperatures above the glass transition (Tg) but below gelatinization (annealing) [22]. Annealing could take place during treatment (incubation step) of these flours facilitating

the interactions between starch chains in the amorphous and crystalline regions which acquired a more stable conformation [23]. Those changes would have allowed the intake of more water inside the granule without breaking it, increasing the value of the peak viscosity. In accordance to these results, Martínez et al. [4] also found a similar pasting profile when flours were subjected to mild extrusion conditions insufficient to induce gelatinization of the flour.

Non-enzymatically treated native sample exhibited the highest peak viscosity and a reduced setback (increase in viscosity during cooling or retrogradation) compared with the native sample. Enzymatically treated (B and BMA) flours showed lower viscosity along the pasting performance and lower setback. The decrease in setback value of treated samples was probably caused by additional interactions between amylose and lipids to form complexes during annealing conditions, which can limit retrogradation in such a way those amylose molecules are no longer available to form double helical structures and retrograde [24]. In fact, Martínez et al. [12] confirmed by X-Ray the presence of a slight sharpening of the peak around 20º, which is related to the formation of V-type complexes, in enzymatically treated flours. Furthermore, taking into account the reduction in the retrogradation trend of B and BMA native flours, it is noteworthy to state that B create products with higher number of branch points that are less prone to short and long term retrogradation [25]. Despite differences encountered in the microstructure appearance of B and BMA flours, practically no differences were shown between their pasting behaviour. This may suggest that changes not only took place on the surface but also in the internal structure. Similar results were found by Dura et al. [15] when treating maize starch with α-amylase, indicating that this enzyme exerted its major action in the inner core and only small holes in the surface were necessary for entering. Even though previous results showed a low susceptibility of native maize flours to the B or BMA catalysis [12], that small accessibility was enough to promote some changes on pasting profiles of the flours.

Pasting profile of extruded flour agreed with mentioned microscopy results, confirming complete starch gelatinization, since no peak viscosity was observed when heating. Instead, an initial peak before heating was displayed, which is in accordance with the great water absorption capacity of these flours. In non-enzymatically treated extruded flour, this peak emerged slow and later, presumably the ethanol used to precipitate treated flours favoured the formation of V-type complexes with amylose or longer chains [12] as previously reported Ao et al. [8]. Those V-type complexes provide starch granules with higher crystallinity and rigidity impeding water absorption. Enzymatically treated (B and BMA) extruded flours presented very low viscosity along pasting profile with no peaks as a result of the hydrolytic activity of the enzyme on the starch chains. In addition, no differences were shown due to the type of enzyme added. It has been reported that the new branched structure of starches treated with branching enzyme leads to weakening of the interactions among amylopectin molecules, giving rise to solutions with relatively low viscosity [26]. The same flat pasting profile was reported when treating extruded flours with amylolytic and cyclodextrin glucanotransferase enzymes [19,27].

3.3 Starch hydrolysis kinetics

The hydrolysis curves of non-extruded and extruded flours are displayed in Fig. 3A and 3B, respectively. Generally, differences in the hydrolysis kinetics of starch have been attributed to the interplay of many factors, such as starch source, granule size, crystallinity, molecular fine structure, surface pores and interior channels among others [11].

For native flours, scarce differences were observed among the hydrolysis curves of treated and non-treated flours. It could only be envisaged that incubated samples seemed more accessible to be hydrolyzed. Large surface area of flour particles increases the water diffusion and enzyme accessibility according to de la Hera et al. [28], which is in agreement with microscopy results encountered in the present study. Thus, the disaggregation of starch granules from the protein matrix would favor the α-amylase accessibility, increasing starch susceptibility to be hydrolyzed. Furthermore, these results confirm that the action of B and BMA on these flours was minimal, owing to the fact that no significant differences were observed due to the enzymes action. Therefore, the small holes observed on the surface of BMA starch granules were not enough to enable enzyme accessibility inside the granule.

On the other hand, extrusion treatment led to a significant increase in the enzymatic hydrolysis curve of flours compared with the un-extruded flours, as has been previously reported [3,4,18]. The rupture of granules promoted by gelatinization makes the starch more accessible, exposing the more enzyme-susceptible interior regions to enzyme attack and facilitating the amylolytic hydrolysis [29,30]. No conclusive results could be extracted from the hydrolysis curves of extruded samples, although it was expected that the increase in the number of short outer chains and branching points of amylopectin induced by B and BMA [12] would hinder pancreatic α-amylase binding to the starchy-substrate. Nevertheless, the reduction in particle size and the more porous structure already observed in section 3.1 could have masked those changes produced in the fine structure.

It must be stressed out that glucose release was gradually increasing for native flours as the reaction proceeded (Fig. 3A), whereas for extruded flours a fast increase of glucose release was observed during the first 10 minutes of reaction, reaching rapidly a plateau (Fig. 3B). Therefore, in extruded flours, all digestible starch was rapidly hydrolyzed within the first minutes of the reaction as a result of; the greater ability of the enzyme to enter into the disrupted structure already shown in the photomicrographs and the higher surface to volume ratio of the particles.

Enzymatic treatment was not enough to promote significant differences on hydrolysis rate and extent (Table 1) as indicated the kinetics parameters obtained by fitting the hydrolysis experimental data to a first-order equation. The end point values (C_∞) obtained in the hydrolysis process reflected the concentration at the equilibrium point. No great differences were obtained for this parameter attending the type of treatment or the type of flour. Indeed, the most significant change in C_∞ was the decrease in this value for native flours after the possible annealing produced during such hydrothermal

conditions. This would suggest that the greater crystalline perfection acquired after annealing could have limited the enzymatic hydrolysis within the 3 hours of incubation.
Regarding the digestibility rate constant (*k*), despite the fact that treated native flours seemed more rapidly digested compared to non-treated sample, significant differences were only found due to the type of flour. Extruded flours presented the highest hydrolysis rates compared to native flours. Butterworth et al. [31] indicated that significant differences in *k* are indicative of structural differences. Furthermore, low *k* values have been reported when there was a slow diffusion of pancreatic amylase into the starch granule as digestion proceeded [32]. In this research, the lowest values of *k* found for all native samples, indicated their minor susceptibility to digestion. Therefore, it seems that the structural characteristics of the flour initially employed largely affected the digestion process rather than the ulterior treatment carried out in order to modify this initial structure. Further strategies to diminish the substrate affinity to the binding active-subsites of pancreatic α-amylase should be approach bearing in mind the changes produced at all hierarchical levels in the starch structure of maize flours during such treatments. Results showed that the negative effect of rapid digestion induced by extrusion was not overcome by increasing the branching points and the ratio of short chains to longer chains in amylopectin.
Somehow this can explain the controversy found in the results reported. Thus, some authors [8,9,33] reported that an increase in the proportion of short outer chains of amylopectin and in branching points likely contribute to their slow digestion property. However, Han et al. [34] indicated that short chains of amylopectin and amylose were preferentially digested (rapidly), while DP 121 chains were resistant to amylase hydrolysis, followed by those of DP 46. Furthermore, Zhang et al. [35,36] found that maize starches with either longer or shorter branches showed slower digestion properties based on: 1) the potential of long branches to form crystallites and, 2) the presence of a highly branched amorphous lamella, entailing a higher content in short chains, that halt the rearrangement of the crystalline double helices in amylopectin. In this way, Bertoft et al. [37] recently hypothesized that even those nuances in the structural architecture can have a pronounced effect on the properties of starch.

3.4 Resistant starch content

Resistant starch was significantly higher for native samples and for extruded flour without further treatment (Table 1). Whereas no significant differences were found due to the treatment in native samples, when the extruded sample was treated, weather in presence or in absence of enzyme, the RS content was significantly reduced owing to the incubation step. During treatment of the samples, conditions similar to annealing were produced. Despite the fact that different results have been reported for annealed starches in terms of digestibility, some authors [38,39] found a reduction in the resistant starch content and in the extent of the reaction in pulse and corn annealed starches, respectively. They explained that the increased hydrolysis can be due to the formation of a more porous structure, allowing a greater accessibility of hydrolytic enzyme into the starch interior which may negate the effect of crystalline perfection and starch chain interactions on enzyme susceptibility. In this way, it could be hypothesized that these

structures with a greater crystalline perfection could be sometimes hydrolyzed due to this increased porosity making them more accessible to enzymatic activity, and not contributing to the increase in the RS level. This trend would only be significant in extruded matrices compared with the native ones, being the later less prone to mild hydrothermal reorganizations as a consequence of their packed morphology. It is important to point out that amylose is the main starch molecule related to RS [35]. Martínez et al. [12] reported an increase in the amylose content during the branching enzyme treatments. However, they hypothesized that amylopectin chains would have been the preferable substrate to be cleaved during enzymatic treatment, since amylose was supposedly complexed with other amyloses, polar lipids, or ethanol. Thereby, the decrease in the amount of amylopectin would be responsible for the apparent increase in the amylose fraction.

4 Conclusions

The strategy used to partially shorten the length of exterior branch chains of amylopectin, as well as increase the branch points of extruded maize starch, through branching enzyme and maltogenic α-amylase treatments, did not reduce its overall digestion rate. Results suggested that the effects of resistant molecular and supramolecular structures on starch hydrolysis could have been masked by secondary changes produced at larger hierarchical levels in the starch structure of maize flours during such treatment. All these structural modifications affected the physicochemical properties of maize flours, but in contrast to previous studies, in a different way than they did to starches. Therefore, future comparative studies focused on how those enzymatic treatments differently affect both starches and flours properties could shed some light on optimizing the enzyme substrate (raw material) and the enzymatic conditions to produce ingredients with desirable functional and nutritional properties.

Acknowledgements

The authors acknowledge the financial support of the Spanish Ministry of Economy and Competitiveness (Project AGL2014-52928-C2) and the European Regional Development Fund (FEDER). The authors are also grateful to Molendum Ingredients and Novozymes for supplying the flour and the enzymes, respectively. They also would like to thank Laura Pascual Velasco for the assistance for carrying out the enzymatic treatments. Laura Roman would like to thank the University of Valladolid for her pre-doctoral fellowship.

References

[1] M.E. Camire, A. Camire, K. Krumhar, Chemical and nutritional changes in foods during extrusion, *Critical Reviews in Food Science and Nutrition* 29 (1990) 35-57.

[2] L. Wen, P. Rodis, B. Wasserman, Starch fragmentation and protein insolubilization during twin-screw extrusion of corn meal, *Cereal Chemistry* 67 (1990) 268-275.

[3] M.M. Martínez, A. Calviño, C.M. Rosell, M. Gómez, Effect of different extrusion treatments and particle size distribution on the physicochemical properties of rice flour, *Food and Bioprocess Technology* 7 (2014) 2657-2665.

[4] M.M. Martínez, C.M. Rosell, M. Gómez, Modification of wheat flour functionality and digestibility through different extrusion conditions, *Journal of Food Engineering* 143 (2014) 74-79.
[5] J.D.J. Berrios, J.R. Ascheri, J.N. Losso, Extrusion processing of dry beans and pulses, in: M. Siddiq, M.A. Uebersax (Eds.), Dry Beans and Pulses. Production, Processing and Nutrition, Wiley Blackwell, Iowa, 2013, pp. 185-203.
[6] M. Gómez, M.M. Martínez, Changing flour functionality through physical treatments for the production of gluten-free baking goods, *Journal of Cereal Science* 67 (2016) 68-74.
[7] A. Lovegrove, C.H. Edwards, I. De Noni, H. Patel, S.N. El, T. Grassby, C. Zielke, M. Ulmius, L. Nilsson, P.J. Butterworth, P.R. Ellis, P.R. Shewry, Role of polysaccharides in food, digestion and health, *Critical Reviews in Food Science and Nutrition* 57 (2017) 237-253.
[8] Z. Ao, S. Simsek, G. Zhang, M. Venkatachalam, B.L. Reuhs, B.R. Hamaker, Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure, *Journal of Agricultural and Food Chemistry* 55 (2007) 4540-4547.
[9] Q.T. Le, C.K. Lee, Y.W. Kim, S.J. Lee, R. Zhang, S.G. Withers, Y.R. Kim, J.H. Auh, K.H. Park, Amylolytically-resistant tapioca starch modified by combined treatment of branching enzyme and maltogenic amylase, *Carbohydrate Polymers* 75 (2009) 9-14.
[10] K.H. Park, T.J. Kim, T.K. Cheong, J.W. Kim, B.H. Oh, B. Svensson, Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the α-amylase family, Biochimica et Biophysica Acta (BBA) 1478 (2000) 165-185.
[11] M. Miao, S. Xiong, B. Jiang, H. Jiang, S.W. Cui, T. Zhang, Dual-enzymatic modification of maize starch for increasing slow digestion property, *Food Hydrocolloids* 38 (2014) 180-185.
[12] M.M. Martínez, J. Pico, M. Gómez, Synergistic maltogenic α-amylase and branching treatment to produce enzyme-resistant molecular and supramolecular structures in extruded maize matrice, *Food Hydrocolloids* 58 (2016) 347-355.
[13] U. Uthumporn, Y. Shariffa, A. Karim, Hydrolysis of native and heat treated starches at sub-gelatinization temperature using granular starch hydrolyzing enzyme, *Applied Biochemistry and Biotechnology* 166 (2012) 1167-1182.
[14] AACC. Approved methods of the American association of cereal chemists, 61-02.01 (pasting properties) and 32.40.01 (resistant starch). American Association of Cereal Chemists, St. Paul, Minnesota, 2012.
[15] A. Dura, W. Blaszczak, C.M. Rosell, Functionality of porous starch obtained by amylase or amyloglucosidase treatments, *Carbohydrate Polymers* 101 (2014) 837-845.
[16] I. Goñi, A. Garcia Alonso, F. Saura Calixto, A starch hydrolysis procedure to estimate glycemic index, *Nutrition Research* 17 (1997) 427-437.
[17] M.A. Gularte, C.M. Rosell, Physicochemical properties and enzymatic hydrolysis of different starches in the presence of hydrocolloids, *Carbohydrate Polymers* 85 (2011) 237-244.

[18] X. Zhang, Y. Chen, R. Zhang, Y. Zhong, Y. Luo, S. Xu, J. Liu, J. Xue, D. Guo, Effects of extrusion treatment on physicochemical properties and in vitro digestion of pregelatinized high amylose maize flour, *Journal of Cereal Science* 68 (2016) 108-115.
[19] L. Román, A. Dura, M.M. Martínez, C.M. Rosell, M. Gómez, Combination of extrusion and cyclodextrin glucanotransferase treatment to modify wheat flours functionality, *Food Chemistry* 199 (2016) 287-295.
[20] A.K. Shrestha, C.S. Ng, A. Lopez-Rubio, J. Blazek, E.P. Gilbert, M.J. Gidley, Enzymatic resistance and structural organization in extruded high amylose maize starch, *Carbohydrate Polymers* 80 (2010) 699-710.
[21] G. Angelidis, S. Protonotariou, I. Mandala, C.M. Rosell, Jet milling effect on wheat flour characteristics and starch hydrolysis, *Journal of Food Science and Technology* 53 (2016) 784-791.
[22] C. Biliaderis, Structural transitions and related physical properties of starch, in: J. BeMiller, R. Whistler (Eds.), Starch. Chemistry and Technology, Academic Press, New York, 2009, pp. 293-372.
[23] E.D.R. Zavareze, A.R.G. Dias, Impact of heat–moisture treatment and annealing in starches: A review, *Carbohydrate Polymers* 83 (2011) 317-328.
[24] S.J. Wang, C.L. Li, L. Copeland, Q. Niu, S. Wang, Starch Retrogradation: A Comprehensive Review, *Comprehensive Reviews in Food Science and Food Safety* 14 (2015) 568-585.
[25] W.W. Li, C.M. Li, Z.B. Gu, Y.J. Qiu, L. Cheng, Y. Hong, Z.F. Li, Relationship between structure and retrogradation properties of corn starch treated with 1,4-α-glucan branching enzyme, *Food Hydrocolloids* 52 (2016) 868-875.
[26] M.J.E.C. van der Maarel, Starch-processing enzymes, in: R.J. Whitehurst, M. van Oort (Eds.), Enzymes in Food Technology, Blackwell publishing Ltd., Ames, Iowa, 2010, pp. 320-331.
[27] M.M. Martínez, J. Pico, M. Gómez, Physicochemical modification of native and extruded wheat flours by enzymatic amylolysis, *Food Chemistry* 167 (2015) 447-453.
[28] E. de la Hera, M. Gómez, C.M. Rosell, Particle size distribution affecting the starch enzymatic digestion and hydration of rice flour carbohydrates, *Carbohydrate Polymers* 98 (2013) 421-427.
[29] A. Hagenimana, X. Ding, T. Fang, Evaluation of rice flour modified by extrusion cooking, *Journal of Cereal Science* 43 (2006) 38-46.
[30] A.K. Shrestha, J. Blazek, B.M. Flanagan, S. Dhital, O. Larroque, M.K. Morell, E.P. Gilbert, M.J. Gidley, Molecular, mesoscopic and microscopic structure evolution during amylase digestion of extruded maize and high amylose maize starches, *Carbohydrate Polymers* 118 (2015) 224-234.
[31] P.J. Butterworth, F.J. Warren, T. Grassby, H. Patel, P.R. Ellis, Analysis of starch amylolysis using plots for first-order kinetics, *Carbohydrate Polymers* 87 (2012) 2189-2197.
[32] S. Dhital, A.K. Shrestha, M.J. Gidley, Relationship between granule size and in vitro digestibility of maize and potato starches, *Carbohydrate Polymers* 82 (2010) 480-488.

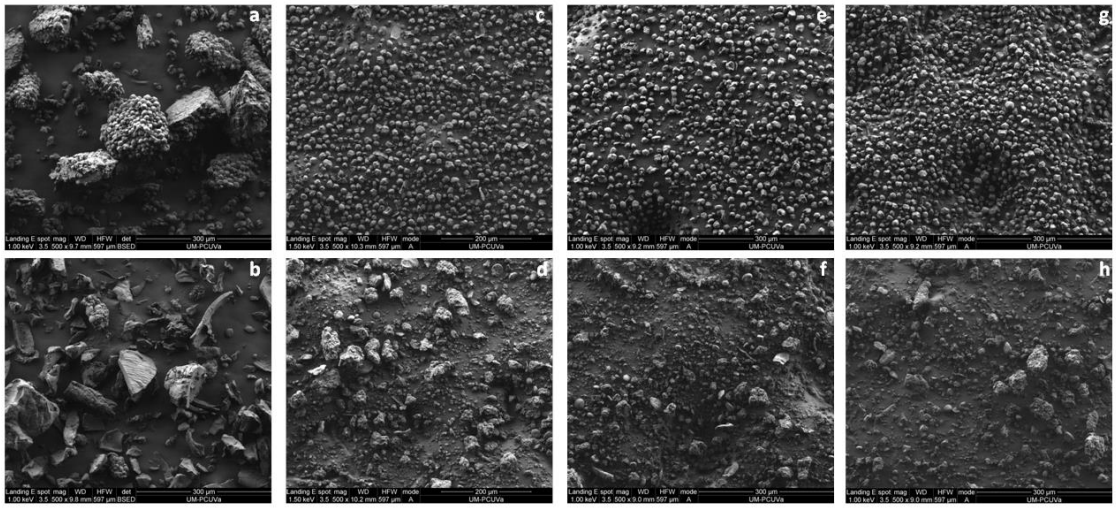
[33] X. Li, M. Miao, H. Jiang, J. Xue, B. Jiang, T. Zhang, Y. Gao, Y. Jia, Partial branching enzyme treatment increases the low glycaemic property and α-1,6 branching ratio of maize starch, *Food Chemistry* 164 (2014) 502-509.
[34] X.Z. Han, Z. Ao, S. Janaswamy, J.L. Jane, R. Chandrasekaran, B.R. Hamaker, Development of a low glycemic maize starch: preparation and characterization, *Biomacromolecules* 7 (2006) 1162-1168.
[35] G. Zhang, Z. Ao, B.R. Hamaker, Nutritional property of endosperm starches from maize mutants: a parabolic relationship between slowly digestible starch and amylopectin fine structure, *Journal of Agricultural and Food Chemistry* 56 (2008) 4686-4694.
[36] G. Zhang, M. Sofyan, B.R. Hamaker, Slowly digestible state of starch: Mechanism of slow digestion property of gelatinized maize starch, *Journal of Agricultural and Food Chemistry* 56 (2008) 4695-4702.
[37] E. Bertoft, G.A. Annor, X. Shen, P. Rumpagaporn, K. Seetharaman, B.R. Hamaker, Small differences in amylopectin fine structure may explain large functional differences of starch, *Carbohydrate Polymers* 140 (2016) 113-121.
[38] H.J. Chung, Q. Liu, R. Hoover, Effect of single and dual hydrothermal treatments on the crystalline structure, thermal properties, and nutritional fractions of pea, lentil, and navy bean starches, *Food Research International* 43 (2010) 501-508.
[39] S. O'Brien, Y.J. Wang, Susceptibility of annealed starches to hydrolysis by α-amylase and glucoamylase, *Carbohydrate Polymers* 72 (2008) 597-607.

Table 1. Kinetic parameters extracted from first-order fitting of the experimental enzymatic hydrolysis data and resistant starch content of flours samples.

Flour	**Treatment**	***k* (min^{-1})**	***C*$_\infty$ (%)**	**RS (%)**
Native	Non-treated	0.002a	50.47c	13.83b
	No enzyme	0.008a	18.41a	12.34b
	B	0.006a	24.00ab	12.04b
	BMA	0.004a	17.68a	13.57b
Extruded	Non-treated	0.461b	25.64ab	13.26b
	No enzyme	0.372b	29.53b	9.66a
	B	0.391b	28.89b	9.33a
	BMA	0.541b	24.52ab	8.86a

Values followed by different letters within a column denote significantly different levels (P<0.05). RS= Resistant Starch. Non-treated= not subjected to any treatment; no enzyme= treatment in the absence of enzyme, B= branching enzyme treatment and BMA= branching enzyme and maltogenic α-amylase treatment.

A



B

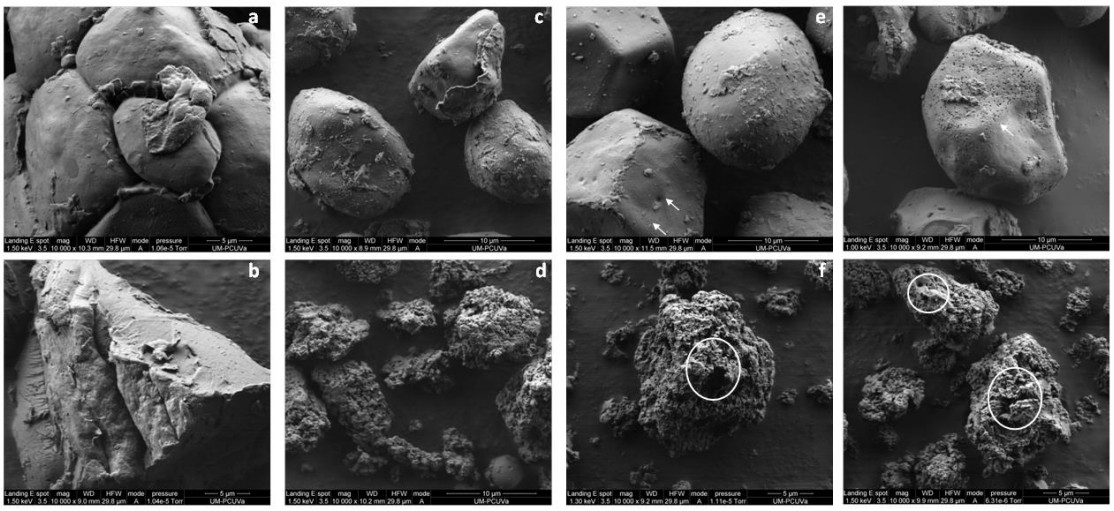


Figure 1. Scanning electron micrographs of native (a, c, e, g) and extruded (b, d, f, h) flours at low (A) and high (B) magnification, respectively. Non-treated (a, b), non-enzymatically treated (c, d), treated with B (e, f) and treated with BMA (g, h) flours. Arrows and circles show holes and cavities, respectively.

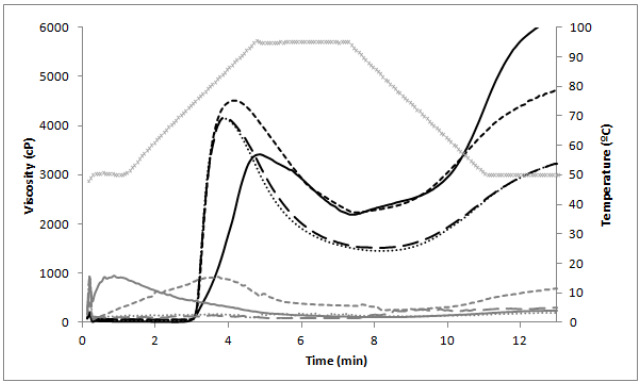


Figure 2. Pasting profiles of native (black) and extruded (grey) flours. Non-treated (continuous line), non-enzymatically treated (short broken line), treated with B (long broken line) and treated with BMA (dot line).

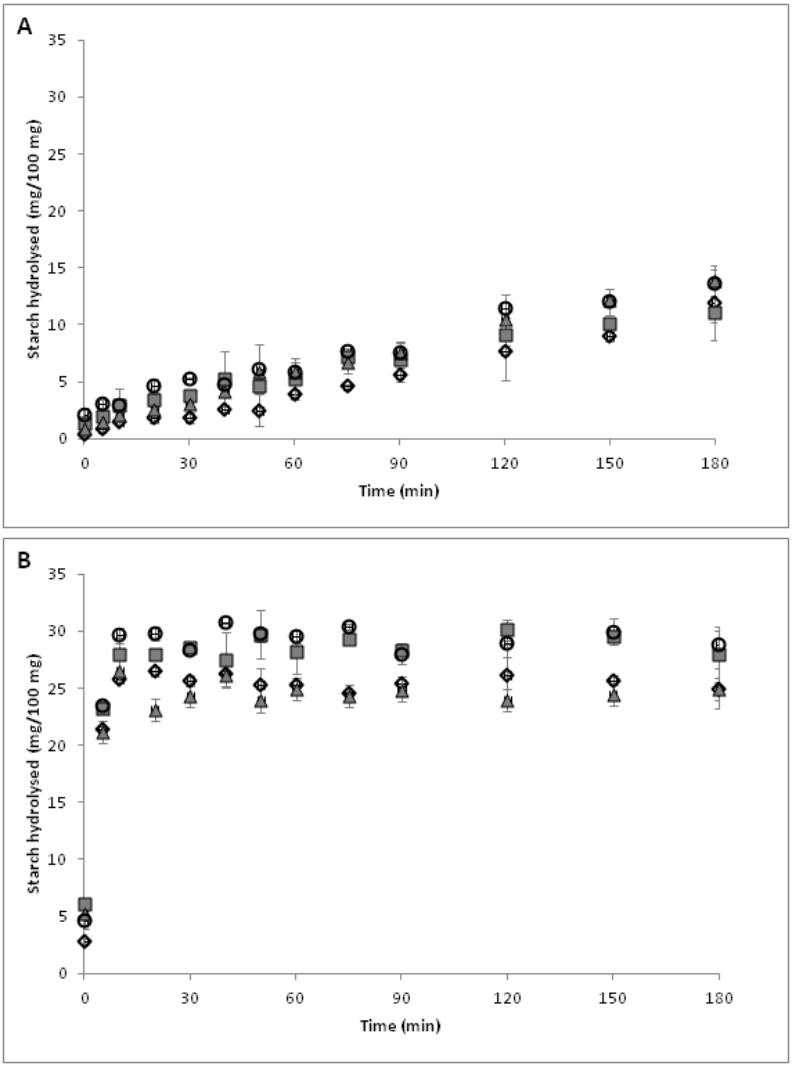


Figure 3. In vitro starch hydrolysis of the native (A) and extruded (B) flours treated with B (■) or BMA (▲) compared with their controls; non-treated (◊) and non-enzymatically treated (○) flours.