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Combination of extrusion and cyclodextrin glucanotransferase treatment to modify wheat flours functionality

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Running title: Physical and enzymatic treatments of flours

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Abstract

This research aims to vary functional properties of native and extruded wheat flours combining cyclodextrin glucanotransferase and extrusion treatments. The level of released cyclodextrins (CD) was assessed, besides the microstructure, crystallinity, pasting properties and starch hydrolysis of the flours. Photomicrographs of enzymatically treated flours suggested the production of fragile structures that broke easily. Enzymatic hydrolysis was significantly higher in extruded flours, as confirmed the CD levels, being predominant the γ-CD followed by α-CD, whereas very low β-CD values were obtained probably due to the formation of CD-lipid complexes, as suggested X-ray diffractometry results. Both extruded and native samples showed very low viscosity and flat pasting profile consequence of the enzyme hydrolytic activity on the starch chains. Enzymatically treated flours (native and extruded) showed higher hydrolysis rates at the early hydrolysis stage, and extruded flours exhibited higher fractal exponent h in agreement with the extended crystalline structures resulting from enzymatic treatment.

Keywords: wheat flour, CGTase, extrusion, cyclodextrin, starch characteristics.

1 Introduction

Starch and starch based products, such as flours, are common raw materials used in food industry because they have unique thermal, structural and functional properties that permit their use in food products and industrial applications. Starch and starch based products can be modified by chemical, physical or enzymatic treatment to improve industrial applications. Physical and enzymatic treatments of these products allow the modification of their nutritional and functional properties. Nevertheless, when enzyme treatment is utilized, native starch is only partially accessible for the enzyme catalysis, thus it is necessary to promote the damage or breakage of the starch granules (Uthumporn, Shariffa, & Karim, 2012). Hydrothermal treatment, such as extrusion, which combines high temperature and pressure, fosters gelatinisation and dextrinization depending on the conditions of the extrusion (Martínez, Calviño, Rosell, & Gómez, 2014). After gelatinisation, starch is more accessible and it is therefore directly available for enzymatic modification (Martínez, Pico, & Gómez, 2015; Patel, Day, Butterworth, & Ellis, 2014).

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an endoenzyme that catalyses four different reactions (hydrolysis, cyclization, coupling and disproportionation) by cleaving α-1,4-glycosidic bonds present in the inner part of a polysaccharide chain (Terada, Yanase, Takata, Takaha, & Okada, 1997). Among these reactions, cyclization is the specific enzymatic reaction that releases cyclic oligomers, known as cyclodextrins (CDs), from starch or starch derivatives (Li, Chen, Gu, Chen, & Wu, 2014). The most common CDs are α-, β-, and γ-CDs (with six, seven, and eight 1,4-linked D-glucose units, respectively), containing trace amounts of CDs with more than nine D-glucose units (Terada et al., 1997). CDs are extensively used in the food industry for different applications such as food additives, encapsulation of molecules

67 (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara, 2009; Astray,
68 Mejuto, Morales, Rial-Otero, & Simal-Gandara, 2010) and as a source of dietary fibre
69 (Artiss, Brogan, Brucal, Moghaddam, & Jen, 2006). The enzyme CGTase has been also
70 proposed to slow down starch retrogradation and staling in starch or flour based
71 products (Gujral, Haros, & Rosell, 2003b; van der Maarel & Leemhuis, 2013) and to
72 improve the quality of bakery products (Gujral, Guardiola, Carbonell, & Rosell, 2003a).
73 Therefore, the modification of starch with CGTase provides modified starches with the
74 additional functionality that offer the released CDs.
75 Even though several studies have been focused on the production of CDs from different
76 tuber and cereal starches (Calsavara, Dias da Cunha, Balbino, Zanin, & de Moraes,
77 2011; Gujral & Rosell, 2004; Yamamoto, Zhang, & Kobayashi, 2000), CDs production
78 from flour has never been attempted. Flour in comparison with starch, contains proteins,
79 lipids, sugars and other non-starchy components. Therefore, enzymatic treatment of
80 flours can possibly be influenced by the interactions between starch and those non-
81 starch components, giving rise to different properties than those of starch. Moreover,
82 flour modification can be a good alternative to starch modification for their use in
83 industrial processes, being economically a more viable process and with lower
84 environmental impact (Eckhoff & Watson, 2009).
85 The objective of this research was to provide wheat flours with diverse functional
86 properties by enzymatic treatments. In pursuing the aim, CGTase was applied to native
87 and extruded wheat flours and the level of released CDs was assessed. In addition,
88 enzymatic treatment was carried out at two ratios of liquid volume to starch mass, given
89 the impact of that ratio on the absorption of the enzyme to the starch surface and also
90 considering the economic impact of drying when industrial application of the process.
91 To determine the functionality of enzymatically treated flours, the microstructure,

crystallinity, pasting properties, hydration properties and digestibility, were also investigated.

2 Materials and methods

2.1 Materials

Native and extruded wheat flours were supplied by Molendum Ingredients (Zamora, Spain). Extrusion of native wheat flour (11.73% of moisture, 11.78% of protein and 4.97% of damage starch contents) was carried out by Molendum Ingredients in a single screw extruder Bühler Basf (Bühler S.A., Uzwil, Switzerland). The length to diameter (L/D) ratio for the extruder was 20:1. The extrusion conditions were carried out based on preliminary experiments in order to ensure starch gelatinization. Wheat flour was extruded at a maximum barrel temperature of 160 ºC and a feed moisture content of 50 L/h with a feed rate of 500 kg/h and a screw speed of 340 rpm. Extruded product was dried by convection air up to 10.40% of moisture content and then ground with a compression roller till particle size was lower than 200 microns.

Cyclodextrin glucanotransferase (CGTase) from *Bacillus licheniformis* Toruzyme® 3.0 L (declared activity: 3.0 KNU/g) was kindly provided by Novozymes (Bagsvaerd, Denmark).

2.2 Methods

2.2.1 Flour measurements

Native wheat flour composition was analyzed following AACC Methods (AACC, 2012) for moisture, method 44-16.01; damaged starch 76-30A; and protein content, method 46-30.01.

2.2.2 Flour modification by CGTase

First, the enzyme solution was prepared by dissolving 41.65 µL ±0.001 µL (0.15 KNU) of CGTase in the appropriate volume of distilled water (40 mL or 80 mL). Then, a pre-weighed amount of starch (10 g) were suspended in 40 mL or 80 mL of enzyme solution to obtain ratios of flour mass to liquid content of 1:4 or 1:8, respectively. Slurries of native and extruded flours were also prepared in 40 mL or 80 mL distilled water without CGTase addition, as control. Flour slurries were well mixed with a glass rod, covered by plastic film to avoid drying of the sample and then incubated at 60 °C for 60 min. During incubation, flour slurries were vigorously stirred each 15 min so as to avoid the flour particles to settle down. To stop the enzymatic reaction and to dry the flour slurries, the pastes were heated at 105 °C for 5 h. Afterwards, samples were rested in a desiccator at room temperature for 3 min, before milling in a Moulinex super juniors (Groupe Seb Iberica, S.A, Barcelona, Spain) for 20 s. Flours were stored in airtight plastic containers perfectly sealed at 4 °C until analysis. Thereby, the whole process of flour hydrolysis was performed considering the feasibility of scaling up the process in the food industry.

2.2.3 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 1.5 KeV in high vacuum mode with a backscattered electron detector (BSED).

2.2.4 Cyclodextrin content of flour samples

Release of the most common CDs; α-CD, β-CD and γ-CD was followed colorimetrically via the formation of inclusion complexes with different organic

142 compounds. The ability of α-CD to form inclusion complex with methyl orange (MO)
143 was tested following the method reported by Lejeune, Sakaguchi and Imanaka (1989),
144 slightly modified. The methyl orange (MO) stock solution was prepared at 5 mM in 50
145 mM sodium phosphate buffer pH 6.0 by agitating at 40 °C. A dilution of 1:50 of MO
146 was prepared, in which final concentration of methyl orange was 0.1 mM. A calibration
147 curve of α-CD was performed in the range 0-1946 μg of α-CD. α-CD in flours were
148 measured by suspending 250 mg in 2.5 mL of 50 mM sodium phosphate buffer, after
149 stirring for five minutes, they were centrifuged at 10,000 x *g* for 10 min. Supernatant (2
150 mL) was mixed with 2 mL MO and two drops of 0.275 N HCl were added. Then,
151 cuvettes were shacked and kept into the fridge for 15 minutes. Optical density was
152 measured at 505 nm in UVmini-1240 spectrophotometer (Shimadzu Corporation,
153 Kyoto, Japan).
154 Concentration of β-CD was analysed following the method described by Goel and Nene
155 (1995) based on the decrease in absorbance at 550 nm due to phenolphthalein-CD
156 complex formation, with slight modifications. A calibration curve of β-CD was
157 performed in the range 0-100 μg. The phenolphthalein solution was prepared at 4 mM
158 in 125 mM Na_2CO_3 buffer pH 10.5. Samples (50 mg) were suspended in 500 μL 50 mM
159 Tris-HCl buffer pH 8.0 and stirred for five minutes. After centrifuging, as was described
160 above, 200 μL of supernatant were mixed with 1 mL phenolphthalein solution and
161 absorbance measured immediately at 550 nm in UVmini-1240 spectrophotometer.
162 γ-CD was determined measuring the colour increase at 630 nm due to the formation of
163 inclusion complexes with bromocresol green (BCG) following the method reported by
164 Kato and Horikoshi (1984) slightly modified. The working BCG solution was prepared
165 by mixing 0.5 mL of 5 mM BCG (in 20% ethanol solution) and 10 mL of 0.2 M citrate
166 buffer pH 4.2. A calibration curve of γ-CD in the range 0-700 μg was performed. Flour

sample (150 mg) was extracted with 1500 μL 0.2 M citrate buffer pH 4.2. Clear supernatant (500 μL) obtained after centrifuging were mixed with 1 mL BCG, after shaking the absorbance was read at 630 nm in a UVmini-1240 spectrophotometer. Experimental results are the average of three replicates.

2.2.5 Flour crystallinity by X-ray diffraction (XRD)

Samples were analysed using a Bruker D8 Discover A25 (Bruker AXS, Rheinfelden, Germany) equipped with a copper tube operating at 40 kV and 40 mA, producing CuKa radiation of 0.154 nm wavelength. Diffractograms were obtained by scanning from 5 º to 40 º (2theta) at a rate of 1.2 º/min, a step size of 0.02 º, a divergence slit width variable (DS) of 5 mm, a scatter slit width (SS) of 2.92 º and a nickel filter to exclude Kβ radiation.

2.2.6 Pasting properties

Pasting properties of flours were determined following the standard method 61.02.01 (AACC, 2012) by a Rapid Visco Analyser (RVA-4C) controlled by Thermocline software (Perten, Uppsala, Sweden) for Windows. RVA measurements were carried out in duplicate.

2.2.7 Gel hydration properties

Water absorption index (WAI), swelling power (SP) and water solubility index (WSI) of the different flours were determined following the method of Toyokawa, Rubenthaler, Powers and Schanus (1989), with the modifications reported by Rosell, Yokoyama and Shoemaker (2011). Firstly, flour (50 ± 1 mg) sample was dispersed in 1.0 mL of distilled water in an eppendorf tube using a wire rod and heated at 90 °C for

10 min in a water bath. The cooked paste was cooled in an ice water bath for 10 min and then centrifuged at 3000 × *g* at 4 °C for 10 min. The supernatant was decanted into an evaporating dish and the residue of the eppendorf tube was weighed (Wr). The weight of dry solids was recovered by evaporating the supernatant at 105 °C till constant weight (Ws). Two replicates were made for each sample. WAI, WSI and SP were calculated as follows:

WAI (g/g) = Wr/Wi

WSI (g/100 g) = Ws/Wi × 100

SP (g/g) = Wr/ (Wi – Ws)

where Wi was the sample weight in dry basis (g, db). Values were the average from two replicates. Moisture content of the flour was analysed according to the method 44-16.01 (AACC, 2012).

2.2.8 *In vitro* starch digestibility

In vitro starch digestibility was measured following the method described by Dura, Blaszczak and Rosell (2014). Experimental data were fitted to a fractal-like first-order kinetic model (Kansou, Buléon, Gérard, & Rolland-Sabaté, 2015) with nonlinear regression of hydrolysis data according to the Weibull model.

$X_t = X_\infty [1\text{-}\exp(-k_w t^{1-h})]$ Eq. 1

where X_t is the concentration of product at time t, X_∞ is the value of extent hydrolysis at t=∞, and k_w is the average reaction rate during first unit of time, and h is an empirical constant called fractal exponent, that describes the reaction rate retardation over time.

2.2.9 Statistical analysis

Differences among results were studied by analysis of variance (one-way ANOVA). Fisher's least significant difference (LSD) was used to describe means with 95% confidence intervals. The statistical analysis was performed with the Statgraphics Centurion XVI software (Statpoint Technologies, Inc., Warrenton, USA).

3. Results and discussion

3.1 Microstructure of flours

The effect of enzymatic treatment of native and extruded flours is shown in Figure 1. Flour samples (native and extruded) were enzymatically treated at two different ratios of flour to liquid volume of 1:4 and 1:8 (g/ml), because Zhang et al. (2012) reported the significant effect of the liquid volume and starch mass ratio on the enzyme adsorption to starch surface and consequently on the enzymatic hydrolysis. Only samples treated at the ratio of flour to liquid volume of 1:8 (g/ml) are displayed because no differences were observed on the microstructure due to the dilution. All samples, even native flour, were subjected to the same treatment in the presence or the absence of enzyme, to eliminate possible responses owing to water suspension and drying processes. Some starch granules (Figure 1, arrow marks) were envisaged in the native flour. The reduced amount of visible intact starch granules in native flour was attributed to the drying process. As was previously mentioned all flours, untreated and enzymatically treated, were subjected to the same process and drying was carried out at high temperature to ensure the inactivation of the enzyme, which might produce starch gelatinization. Neither untreated nor treated extruded flour exhibited starch granules, which agree with the starch gelatinization induced by extrusion (Martínez et al., 2014). Photomicrographs of enzymatically treated flours (both native and extruded flours) (Figure 1 c, d) seemed to display a structure with smaller particle size. Therefore, changes induced by

241 enzymatic treatment led to fragile structures that broke easily when re-milling the dried
242 treated flours. Those breakable structures could result either from the structural
243 interference of the hydrolysis products or the enzymatic action on the starch structure.
244 In fact, Tian et al. (2009) found lower hardness in the gels obtained from pregelatinized
245 starch in the presence of β-CD and Uthumporn et al. (2012) reported a reduction in the
246 size of the particles due to the extensive degradation of starch granules during
247 hydrolysis. Therefore, the degradation of the starch promoted by CGTase treatment
248 induced brittle structures. Overall, micrographs analysis suggests that CGTase treatment
249 mainly affected internal structure of flours, not being observable changes in the outer
250 structure.
251
252 **3.2 Cyclodextrin content of flour samples**
253 Table 1 shows the amount of α-, β-, and γ-CD released during enzymatic treatment of
254 the different flours. In the absence of the CGTase, independently of the flour to liquid
255 ratio, no CDs were detected, with the exception of γ-CDs detected in the extruded flour,
256 which might suggest that extrusion treatment promotes a slight dextrinization of starch
257 (Colonna, Doublier, Melcion, Demonredon, & Mercier, 1984). In the CGTase treated
258 flours, the three CDs were produced, thus enzymatic hydrolysis occurred in native and
259 extruded flours. Although CGTase was not expected to act on native flour, owing the
260 compact structure of native starch granules (Uthumporn et al., 2012), the gelatinization
261 taking place during the initial stages of the drying process might facilitate the enzymatic
262 action during certain time before CGTase inactivation, leading to the release of CDs in
263 native flours. In addition, that effect was higher when lower ratio flour mass to liquid,
264 which seems to favour the absorption of the enzyme to the starch. Yamamoto et al.
265 (2000) reported that CDs were produced more slowly from intact starch than from heat

266 treated starch at the early stage, and adsorption of CGTase on intact starch granule
267 might retard its successive attacks on neighbouring granules.
268 In extruded flours, the lowest content was observed in β-CD. It has been reported that
269 the reaction temperature affected the yield of β-CD production by CGTase (Kim, Kim,
270 & Lee, 1995) and the amount of β-CD produced at 65 ºC was lower than that produced
271 over 70 ºC. Therefore, the temperature used for flour treatment did not favour the
272 production of β-CD, and the possible formation of CD-lipid complexes should not be
273 disregarded, since having flour as substrate opens that possibility. The highest CD
274 levels were observed for γ-CD, which agrees with the hydrolysis pattern of the CGTase,
275 because this enzyme produces predominantly α-CD in the earlier stage of reaction but
276 with prolonged reaction time the amount of the other CDs can exceed α-CD (Hedges,
277 2009). CGTase reaction occurred easily in extruded flours mainly leading to the
278 formation of γ-CD. The extrusion treatment increased the susceptibility of flours to be
279 attacked by the CGTase, as occurred with other amylolytic enzymes (Martínez et al.,
280 2015). In fact, Alves-Prado et al. (2008) stated that the gelatinization process of starch
281 was a main player with regard to CD production process. In addition, there are many
282 variables and factors to take into account for CDs production from CGTase activity,
283 enzyme origin, sample source and the condition of the reactions can greatly influence
284 the action of the enzyme (Biwer, Antranikian, & Heinzle, 2002). For instance,
285 Calsavara et al. (2011) using the same enzyme (Toruzyme®) to produce CD from corn
286 starch granules found that α- CD had the highest yield, followed by β-, while γ- was
287 hardly obtained.
288
289 **3.3 X-ray diffractometry**

290 Crystallinity of native and extruded wheat starch was observed using XRD (Fig. 2). No
291 clear differences were observed regarding the ratio of flour mass to liquid. Therefore,
292 possible differences induced by the absorption of enzyme to the granules structure
293 seems to be minor in comparison with the effect of starch state in the native and
294 extruded samples. The A-type pattern observed in the original native flour, typical of
295 cereal starches, was lost for all the native flours after drying process (Fig. 2A). Thus,
296 non-enzymatically treated native samples exhibited a V-type crystalline peak at 2θ of
297 around 20°. Non-enzymatically treated extruded flour samples showed two V-type
298 crystalline peaks at 2θ of around 13° and 20°, which were slightly increased as
299 compared to the original extruded flour (Fig. 2B). López-Rubio, Flanagan, Gilbert, &
300 Gidley (2008) affirmed that these V-type crystalline structures can be originated from
301 single helical amylose, such amylose-lipid complexes. Amylose-lipid complexes are
302 hardly observed in raw starch and are generally produced after gelatinization of starch,
303 which occurred during extrusion (Chanvrier et al., 2007). In native flours, amylose-lipid
304 complexes formation could have taken place during the drying process, where, to a
305 certain extent, starch gelatinization is produced as exhibited the SEM micrographs.
306 Regarding CGTase treatment, only a slight increase of the peak around 20° was
307 displayed for native treated samples. Whereas a noticeable increase in the intensity
308 together with a shift of the d-spacing of V-type peaks (13° and 20°) was observed for
309 extruded treated samples. It has been reported that CDs could disrupt the formation of
310 amylose-lipid complex and compete with amylose to form CD-lipid inclusion
311 complexes (Gunaratne & Corke, 2007; Tian et al., 2009, 2010). Furthermore, Tian et al.
312 (2009, 2010) found a more evident V-type crystalline structure formation when β-CD
313 was added to gelatinized and/or retrograded starches, suggesting that this CD is
314 prompted to interact with amylose to form amylose-β-CD-lipid complexes. Therefore,

315 the changes produced on V-type structures during CGTase treatment could be attributed
316 to the interactions of the several CDs with the amylose-lipid complexes previously
317 formed during the drying process and especially during extrusion. These new amylose-
318 CD-lipid complexes could possess different crystalline lattice with a different d-spacing,
319 modifying the intensity and 2θ angle of V-type peaks. Jane (2009) reported that the
320 structure of single-helical complex (V-complex) resembles to that of a CDs-guest
321 molecule complex in which the linear portion of the starch molecule has its hydrophobic
322 side of the molecule facing the cavity of the helix and interacting with the non-polar
323 moiety of the complexing agent.
324 In extruded flour samples, the height of 17.1 and 22.5 peaks seemed to be more
325 pronounced after CGTase treatment, whereas in native flours these differences were not
326 noticeable. Less-organized amorphous regions are primarily susceptible of enzymatic
327 attack (Uthumporn et al., 2012), as a result of the main decrease in these amorphous
328 areas a logical increase of the crystalline peaks appears to be more visible. The more
329 clear increase of these peaks in extruded flours could be due to the more susceptibility
330 of their gelatinized starch to the CGTase treatment, in agreement to the CD content
331 previously described. Therefore, the amylose and amylopectin chains of lower
332 molecular weight generated during extrusion (Colonna et al., 1984), as well as the linear
333 dextrins resulted from the hydrolytic activity of the CGTase (Hedges, 2009) might
334 promote some aggregation or reorganization of their linear chains gaining crystalline
335 order during drying and further storage. In fact, linear starch chains obtained in other
336 enzymatic treatments are reported to possess higher mobility and can provide ordered
337 alignment leading to chains aggregation into crystalline structures (Cai, Shi, Rong, &
338 Hsiao, 2010; Kiatponglarp, Tongta, Rolland-Sabaté, & Buléon, 2015).
339

340 **3.4 Pasting properties**

341 Pasting profile of studied flours is shown in Fig. 3. Not enzymatically treated native and
342 extruded flour showed lower viscosity profile than their original counterparts, which
343 confirm that some gelatinization occurred during drying process. In the case of native
344 untreated samples, drying promoted the partial gelatinization of starch granules,
345 principally with the highest ratio of flour mass to liquid content, declining their peak
346 viscosity, which is in accordance with microscopy results. Furthermore, both
347 breakdown (drop of viscosity during holding at 95 ºC) and setback (increase of viscosity
348 during cooling) were also reduced. Meanwhile, extruded untreated samples, whose
349 starch was previously gelatinized and had high viscosity in cold solution, lost its cold-
350 water absorption capacity after drying process, which seems to be related to the high
351 crystalline peaks observed in extruded treated flours by X-ray. Therefore, an increase of
352 temperature is necessary to break down the crystalline structures, which impede water
353 absorption and delay the increase of viscosity (Sun, Han, Wang, & Xiong, 2014).
354 With regard to enzymatic treatment, both extruded and native samples showed very low
355 viscosity and flat pasting profile with no peak viscosity as a result of the hydrolytic
356 activity of the enzyme on the starch chains. In addition, no differences were shown due
357 to the diverse ratio of flour mass to liquid. Similarly, Gujral and Rosell (2004) reported
358 a decrease in the peak viscosity when CGTase was added to starch suspension,
359 attributing some of the changes in the pasting properties to the released CDs, which
360 could form complexes with different compounds of the flour, for instance, the lipids. In
361 fact, Gujral et al. (2003a) found that the addition of CGTase in the presence of oil
362 produced a marked decrease in the final viscosity and setback, which indicated the
363 interaction between lipids and CDs, which also agrees with X-ray diffractograms.
364

365 **3.5 Gel hydration properties**

366 Gel hydration properties are summarized in Table 2. In general, enzymatically untreated
367 samples displayed higher value for WAI and SP than enzymatically treated samples,
368 and no significant differences were observed due to the ratio flour mass to liquid.
369 Actually, Uthumporn et al. (2012) found less swelling when hydrolyzing corn starch,
370 attributing this to the fact that the amylose located in the amorphous region was
371 extensively degraded and the granule could not swell to its maximum capacity. These
372 results are in agreement with the pasting profile of these flours, since enzymatically
373 untreated flours displayed higher viscosity, which is related to higher absorption
374 capacity and swelling of the starch. In extruded flours, where the action of the enzyme
375 was more intense due to previous gelatinization of the starch, WAI and SP was lower
376 than in the enzymatically modified native flours. That result could be ascribed to the
377 lower action of the CGTase on native flours where the starch was less susceptible to
378 enzyme attack. Regarding WSI, enzymatic treatment of flour as well as the ratio flour
379 mass to liquid content led to higher solubility in extruded flour. Probably, due to its
380 stable semicrystalline structure, starch granules are not soluble in water at room
381 temperature (Jane, 2009). After CGTase treatment, starch could be degraded to low
382 molecular weight carbohydrates, which could contribute to increase the solubility. In
383 fact, the hydrophilic outer structure of the CDs makes them water-soluble (Li et al.,
384 2014). In addition, lipids could have been incorporated into the hydrophobic central
385 cavity of the CDs, leading to changes in the physical properties of these hydrophobic
386 guest molecules, such as an improvement of their water solubility (Messner, Kurkov,
387 Flavia-Piera, Brewster, & Loftsson, 2011).

388

389 **3.6 Starch hydrolysis**

390 Enzymatic *in vitro* hydrolysis was carried out with the aim to determine the
391 susceptibility of those enzymatically treated flours to the enzymatic digestion. The
392 digestibility curves of the enzymatically treated flours besides their respective no treated
393 flours are displayed in Fig. 4. Differences were observed between treated and untreated
394 flours. For native flours it seems that the ratio of flour mass to liquid content affected
395 slightly their susceptibility to enzymatic digestion. Enzymatically treated native flours
396 showed higher hydrolysis rates at the early hydrolysis stage compared to non treated
397 native ones. As it was shown by ESEM, enzymatically treated samples had smaller
398 particle size promoted by the extensive hydrolysis degradation by CGTase, increasing
399 the surface area available for enzyme attack. The starch granules became more
400 accessible to enzyme hydrolysis. It has been reported that damaged starch granules in
401 flour had greater enzymatic digestibility than intact native starch granules and starch
402 digestibility of flours from milled cereal grains increases with the decreasing flour size
403 (Li et al., 2014).
404 Regarding extruded flours, a more erratic behaviour was observed and differences
405 between untreated flours and enzymatically treated flours (Fig. 4A) were not as evident
406 as in the case of native flours (Fig.4B). Extruded flours showed greater susceptibility to
407 be digested, although those enzymatically treated displayed lower maximum hydrolysed
408 starch. As earlier mentioned, the hydrolysis of a significant amount of amylose and
409 amylopectin chains due to the CGTase activity might promote some aggregation or
410 reorganization of the remnant linear chains gaining crystalline order, as it is shown in
411 XRD results. This reorganization might decrease the accessibility of the enzyme to
412 accomplish the starch hydrolysis.
413 Kinetics parameters obtained by fitting the hydrolysis experimental data to the Weibull
414 model are presented in Table 2. The significance of the fractal-like kinetics is assessed

415 through the reaction rate coefficient (k_w) over time and the values of the parameter h.
416 Reaction rate coefficient k_w values are related to the substrate availability for the
417 enzyme to digest. Low k_w values have been reported when there was slow diffusion of
418 pancreatic amylase into the starch granule as digestion proceeds (Dhital, Shrestha, &
419 Gidley, 2010). Thus, the lower k_w values obtained with the enzymatically untreated
420 flours (native and extruded) indicated that they were less susceptible to the digestion
421 and opposed better restriction to the α-amylase action.
422 The fractal exponent h describes the reaction rate retardation over time, and lower
423 values indicate a more exponential curve (Kansou et al., 2015). Enzymatically treated
424 flours showed higher h, which was even greater in the case of extruded flours, thus
425 greater decrease of the reaction rate over the time. This result agrees with previously
426 suggested crystalline structures resulting from enzymatic treatment, which can entail
427 difficulty or even impossibility for α-amylase to penetrate within some granules.
428 Overall, higher values of k_w, followed by higher values of h were obtained for native
429 and extruded samples enzymatically treated with CGTase as compared with their
430 counterparts without enzyme treatment. Nevertheless, the value of hydrolysis extent at
431 time infinite, X_∞, did not follow a clear trend related to the enzyme treatment.
432

433 ## 4 Conclusions

434 This research aimed to vary functional properties of wheat flours combining enzymatic
435 and physical treatments. Thus, cyclodextrin glucanotransferase (CGTase) was applied to
436 native and extruded wheat flours. Photomicrographs of enzymatically treated flours
437 suggested the production of fragile structures that broke easily. Therefore, the
438 degradation of the starch promoted by CGTase treatment induced brittle structures, as a
439 result of either the structural interference of the hydrolysis products or the enzymatic

action on the starch structure. Enzymatic hydrolysis occurred in native and extruded flours, although the degradation extent was significantly higher in extruded flours. In extruded flours, the lowest content was observed in β-CD, possibly due to the formation of CD-lipid complexes with the flour lipids, which were in agreement with X-ray diffractograms. Pasting parameters were significantly affected by enzymatic treatment being more extensive in the extruded flours. Flour modification by the enzyme resulted in a decrease in the maximum viscosity during heating as well as the reduction of swelling power and water absorption index. Enzymatically treated flours (native and extruded) showed higher hydrolysis rates at the early hydrolysis stage, and the extruded flours exhibited higher fractal exponent *h* in agreement with the extended crystalline structures resulting from enzymatic treatment. Overall, CGTase was able to modify flour functionality regarding microstructure, pasting, composition, starch crystallinity and its susceptibility to *in vitro* hydrolysis; and the magnitude of the modification was enhanced by extrusion process facilitating the action of the enzyme.

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583

584 **Tables**

585 **Table 1.** alpha, beta and gamma cyclodextrin content in native and extruded flours.

Type	**Enzyme content (µL)**	**Flour mass: liquid (w/w)**	**α (mg/100g)**	**β (mg/100g)**	**ϒ (mg/100g)**
Native	0	4:1	0	0	0
	0	8:1	0	0	0
	40	4:1	413a	627c	890a
	40	8:1	448a	462b	0
Extruded	0	4:1	0	0	868a
	0	8:1	0	0	863a
	40	4:1	858b	286a	5440b
	40	8:1	858b	286a	6140c

586 Values followed by different letters within a column denote significantly different levels

587 ($P < 0.05$).

588 **Table 2.** Gel hydration properties and kinetic parameters of the enzymatic hydrolysis, extracted from the fitting of hydrolysis data to fractal-like

589 first-order kinetic model, of different treated flours.

Type	**Enzyme content (µL)**	**Flour mass: liquid (w/w)**	**WAI (g/g)**	**WSI (g/100g)**	**SP (g/g)**	k_w	h	X_∞
Native	0	4:1	7.53e	9.54ab	8.33bc	0.04ab	0.13b	51.8a
	0	8:1	7.54e	8.73ab	8.26bc	0.01a	0.14b	118.08c
	40	4:1	3.23c	56.20d	7.39b	0.13d	0.32d	69.04b
	40	8:1	3.14c	58.55d	7.64b	0.14d	0.33d	72.28b
Extruded	0	4:1	8.29f	6.99a	8.92c	0.05b	0.26c	72.63b
	0	8:1	7.16d	13.85b	8.31bc	0.02a	-0.062a	73.28b
	40	4:1	2.89b	46.27c	5.37a	0.08c	0.57e	108.64c
	40	8:1	2.56a	56.85d	5.96a	0.27e	0.68f	78.33b

590 Values followed by different letters within a column denote significantly different levels ($P < 0.05$)