Influence of amyloglucosidase in bread crust properties

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22 **Abstract**

23 Enzymes are used in baking as a useful tool for improving the processing behavior or
24 properties of baked products. A number of enzymes have been proposed for improving
25 specific volume, imparting softness or extend the shelf life of breads, but scarce studies
26 have been focused on bread crust. The aim of this study was to determine the use of
27 amyloglucosidase for modulating the properties of the bread crust and increase its
28 crispness. Increasing levels of enzyme were applied onto the surface of two different
29 partially bake breads (thin and thick crust bread). Amyloglucosidase treatment affected
30 significantly (P<0.05) the colour of the crust and decreased the moisture content and
31 water activity of the crusts. Mechanical properties were modified by amyloglucosidase,
32 namely increasing levels of enzyme promoted a decrease in the force (Fm) required for
33 crust rupture and an increase in the number of fracture events (N_{wf}) related to crispy
34 products. Crust microstructure analysis confirmed that enzymatic treatment caused
35 changes in the bread crust structure, leading to a disruption of the structure, by
36 removing the starchy layer that covered the granules and increasing the number of
37 voids, which agree with the texture fragility.

38

39 **Key words**: bread crust; amyloglucosidase; colour properties; water activity;
40 puncturing; microstructure.

41 **1) INTRODUCTION**

42 Bread is considered worldwide a staple food; being one of the most important sources
43 for human nutrition that provides starch and complex carbohydrates, proteins, minerals
44 and vitamins (Rosell, 2007; 2011). Current consumption trends show that consumers
45 demand fresh bread all day along and freshness is pointed as an essential attribute
46 (Heenan et al., 2008). Fresh bread usually presents an appealing brownish, crispy crust,
47 besides a pleasant aroma and a soft and elastic crumb texture. Nevertheless, those
48 attributes, particularly the crust crispiness, are very rapidly lost. Crust refers to the part
49 of bread near the surface, which is formed during the final baking. Crust has very low
50 water content (Wählby & Skjöldebrand, 2002), because of that it is relatively dry, crisp
51 and brittle in the fresh state (Hug-Iten et al., 2003). Water migration from the crumb and
52 the atmosphere surroundings to the crust induces a transition from the glassy to the
53 rubbery state of the main crust macromolecules (Gondek et al., 2006; Jakubczyk et al.,
54 2008; Van Nieuwenhuijzen et al., 2008; Castro-Prada et al., 2009; Arimi et al., 2010).
55 As consequence, the mechanical properties of the crust associated to crispness changes
56 and crust becomes very soft and leathery (Roudaut et al., 1998), which causes
57 consumer's rejection.

58 Texture has been widely used for assessing bread freshness either by determining crumb
59 hardness or crust crispiness, both of those directly related to bread acceptability. Texture
60 of the bread crust is an important parameter used to define the quality of crispy breads
61 and their freshness, in which multiple sensations involving numerous physical
62 parameters, combining molecular, structural and manufacturing process are implicated
63 (Roudaut et al., 2002, Luyten et al., 2004). Crispy bread crust is originated when starch
64 and gluten matrix are in glassy state and it has been associated with low moisture
65 content and water activity (Stokes & Donald, 2000). Different methods have been

66 proposed for assessing the mechanical properties of the bread crust, although punching
67 is a common feature in all of them. Recently, Altamirano-Fortoul et al. (2013) defined
68 the optimal punching settings for assessing the crust mechanical properties providing
69 information about the internal cell structure. Their results were also supported by water
70 activity and moisture content determinations, and scanning electron microscopy of the
71 crust section, which confirmed the reliability of the mechanical parameters.
72 Although very much attention has been paid to bread crumb and alternatives to soften it,
73 scarce information has been reported about the crust behavior and how to modulate its
74 mechanical properties. Primo-Martín et al. (2006) reported the effect of different
75 enzymes (endoprotease, transglutaminase, alpha-amylase), sprayed onto the dough
76 surface, as possible strategy for extending crust crispiness. Those authors observed that
77 protease activity led to crust with lower water content, stating that protein network has a
78 main role in the crispness perception. In order to control crust moisture diffusion and
79 water uptake to preserve crispy texture, some attempts using hydrocolloids and enzymes
80 have been reported (Altamirano-Fortoul & Rosell, 2010; Rosell et al., 2011).
81 Nevertheless, the possible role of the starch on the mechanical texture of crust is far
82 from understood.
83 Amyloglucosidase is also used in bakery applications, because its hydrolytic activity on
84 starch yields faster fermentations (Sharma & Singh, 2010), improved bread crust colour
85 (Van Oort, 2010), and enhances flavor in crackers (Heiniö et al., 2012). Also, this
86 enzyme is suggested to delay bread staling due to decreasing retrogradation of
87 amylopectin (Würsch & Gumy, 1994). In fact, anti-staling effects of amyloglucosidase
88 in baking are claimed in some patents (Vidal & Guerrety, 1979; Van Eijk, 1991; Van
89 Benschop et al., 2012).

The aim of the present research was to determine whether amyloglucosidase could be used to modulate the mechanical properties of bread crust. For that purpose, the effect of different concentrations of amyloglucosidase on the physicochemical, mechanical properties and the crust structure were tested. Enzyme solutions were applied onto the surface of two specialties of partially baked bread and the crust features of the full baked breads were assessed. Scanning electron microscopy (SEM) of the crust section was used to confirm the reliability of the mechanical parameters.

2) MATERIALS AND METHODS

Two different specialties of part-baked frozen breads provided by Forns Valencians S.A. (Valencia, Spain) were used. Those specialties were selected for giving breads with different crust section, thus hereafter they will be mentioned as thin and thick crusts. Chemical proximate composition of bread with thin crust was 30.1 g/100g moisture content, 60 g/100g carbohydrates (calculated by difference), 2.74 g/100g fats and 6.41 g/100g proteins. The composition of the bread with thick crust was 34.3 g/100g moisture content, 59 g/100g carbohydrates (calculated by difference), 0.72 g/100g fats and 5.41 g/100g proteins.

A food grade commercial amyloglucosidase from *Aspergillus niger (*Amyloglucosidase 1100BG, 1100AGU/g) was provided by Novozyme A/S (Bagsvaerd, Denmark).

Enzymatic treatments

Amyloglucosidase was used to selectively modify the crust starchy components. Enzymatic solutions were prepared by suspending the commercial enzyme in distilled water at the levels described in Table 1.

115 Full baking process and storage

116 Part-baked breads were taken from the freezer (-18ºC) and were placed at room
117 temperature. Loaves were spread evenly with enzymatic solutions over the top surface
118 before baking. The amount of enzyme solution (2 ml) used per piece of bread was
119 sufficient to cover the whole top surface (118.3± 1 cm^2). Dosages were calculated based
120 on previous studies (Altamirano-Fortoul & Rosell, 2010). Control bread was similarly
121 treated but without enzyme. Loaves were thawed at room temperature till the center of
122 the loaf reached 5ºC. After thawing, loaves were baked off in a forced convection oven
123 (Eurofours, Gommegnies, France). Baking conditions varied with specialty and were as
124 follows: 180° C for 11 min in the case of bread with thick crust, 180° C during 16 min
125 for the one with thin crust. Both specialties required a preheated oven at 220°C. For
126 each specialty, three sets of samples were performed for each treatment, which were
127 baked in separate days.

128 Fresh loaves (0.5 h after baking) were tested for textural characteristics (mechanical
129 properties), water activity, moisture content, crust section, crust colour and structure.

130

131 Physicochemical analysis

132 Moisture content and water activity were determined in the crust and crumb of breads.
133 Crust and crumb were separated using a razor blade based on white versus brown
134 colour.

135 Moisture content was determined following ICC standard method (1994) (ICC 110/1).
136 Water activities were measured using a water activity unit (Aqua Lab Series 3, Decagon
137 Devices, Pullman, USA) at 25°C. Crust section analysis was performed by scanning
138 cross section of bread sample, 10 mm thick, in a flat bed scanner equipped with the
139 software HP PrecisoScan Pro version 3.1 (HP scanjet 4400C, Hewlett-Packard, USA).

The default settings for brightness (midtones 2.2) and contrast (highlights 240, midtones 2.2, shadows 5) of the scanner software were used for acquiring the images. The crust section was calculated from the scanned samples at the upper and bottom side using an image analysis program (UTHSCSA Image Tool software, TX, USA).

Colour parameters of bread crust were measured at three different locations by using a Minolta colorimeter (Chroma Meter CR-400/410, Konica Minolta, Japan) after standardization with a white calibration plate (L^* = 96.9, a^* = -0.04, b^* = 1.84). The colour was recorded using CIE-L^* a^* b^* uniform colour space (CIE-Lab) and D65 illuminant, where L^* indicates lightness, a^* indicates hue on a green (-) to red (+) axis, and b^* indicates hue on a blue (-) to yellow (+) axis. The results were reported in the form of total colour difference using Eq (1).

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Eq. (1)}$$

Where: ΔL^*, $\Delta a^*$and Δb^* are the differences between the L^*, a^* and b^* values of the sample and white plate calibration.

Crust darkness was determined on as 100-L^* (100-L^* =0, white and 100-L^* = 100, black) (Sahlström & Brathen, 1997).

Three measurements were performed in each bread and three breads from each treatment were used for this determination. Crust samples were freeze dried and kept for further microstructure studies. Preliminary tests were carried out to confirm that freeze drying was not affecting the crust microstructure.

164 Puncture tests

165 Loaves were puncture tested using a texture analyzer with a 5 kg load (TA XTplus,
166 Stable Micro Systems, Surrey, UK). The analysis consisted in recording the force
167 required to penetrate the bread crust by punching the sample at three different locations:
168 in the middle of the crust area and at 2 cm distance on both sides. Experiments were
169 carried out using two distinct cylindrical probes: 2 mm diameter (punching area=3
170 mm^2) at 0.5 mm/s, and 6 mm diameter (punching area= 28 mm^2) at crosshead speed 40
171 mm/s, following the settings suggested by Altamirano-Fortoul et al. (2013).

172 The data were analyzed using the method proposed by Van Hecke et al. (1998). This
173 method is based on the peak analysis of the force-deformation curves. From the force-
174 deformation curve recorded, the following puncturing parameters were determined:

175 Average puncturing force: $$Fm(N) = \frac{A}{d}$$ Eq. (2)

176 Spatial frequency of structural ruptures: $$N_{wr}\left(m^{-1}\right) = \frac{No}{d}$$ Eq. (3)

177 Average specific force of structural ruptures: $$f_{wr}(N) = \Sigma \frac{\Delta F}{No}$$ Eq. (4)

178 Crispness work: $$W_c(N.m) = \frac{Fm}{N_{wr}}$$ Eq. (5)

179

180 Where: *No* is the total number of peaks, *d* is the distance of penetration (mm), ΔF is the
181 individual force drops for each peak (N) and *A* is the area under the force-deformation
182 curve.

183 Four breads from each set were used for carrying on the puncture test, obtaining 24
184 individual measurements for each experimental point.

185 SEM of bread crust

186 Scanning electron microscopy was used to examine the crust of bread. Slices of bread
187 were freeze-dried previously to the microscopy analysis. Sample cubes (1 cm^3) were
188 fixed with the aid of colloidal silver and then coated with gold (Baltec SCD005) at 10^{-2}
189 Pa and an ionisation current of 40 mA. The observation was carried out in a JEOL JSM-
190 5410 (Jeol, Tokyo, Japan) scanning electron microscope at 10 kV.

191

192 Statistical analysis

193 Data were presented as mean of sample sets. Statistical analysis of the results was
194 performed using Statgraphics Plus V 7.1 (Statistical Graphics Corporation, UK). In
195 order to assess significant differences among samples, a multiple sample comparison
196 was performed. The analysis of variance was carried out to decompose the variance of
197 the data into two components: a between-group component and a within-group
198 component. When the *P*-value of the *F*-test was less than 0.05, there was statistically
199 significant difference between the means of the 2 groups at the 95.0% confidence level.
200 Multiple range test was used to determine which means were significantly different
201 from each other and Fisher's least significant difference (LSD) procedure was used to
202 discriminate among the means. A multifactor analysis of variance was performed to
203 determine which factors have a statistically significant effect on mechanical parameters.
204 Pearson product moment correlations between each pair of variables were also carried
205 out.

206

207 **3) RESULTS AND DISCUSSION**

208 Effect of enzymatic treatments on the physicochemical properties of bread crust

209 Amyloglucosidase was sprayed onto the surface of frozen partially baked breads and the
210 effect of increasing levels of enzyme on the physical and chemical properties of bread
211 crusts was studied. Two different bread specialties with diverse crust thickness were
212 selected for determining the ability of the enzyme to penetrate through the crust. The
213 upper crust of the sample identified as thick crust had a section of 5.10 mm, which was
214 significantly (P <0.05) different than that in the bread with thin crust (2.94 mm).
215 The values obtained for bread crust colour, water activity and moisture content are
216 shown in Table 2. The enzyme concentration had a significant effect on bread crust
217 colour parameters and crust darkness. Comparing breads without enzymatic treatment,
218 the thick crust showed higher L^* (lightness) and lower a^* (redness), with no significant
219 differences on b^*. Those differences could be derived from their different composition
220 and/or processing conditions. In general, regardless the type of crust, the enzymatic
221 treatment decreased lightness, and larger effect was observed on the thick crust that
222 underwent a drop in L^* with the presence of the lowest level of amyloglucosidase.
223 Regarding a^*, the value slightly increased in the thick bread crust due to the addition of
224 amyloglucosidase, but no trend was observed in the case of thin bread crust. In both
225 samples, the b^* value decreased due to the enzyme activity.
226 Concerning to the total colour difference (ΔE) (Table 2), in general, the enzymatic
227 treatment induced a progressive increase of the values of this parameter when increasing
228 the enzyme concentration, with the exception of the thin crust sample treated with
229 250mg/10ml amyloglucosidase. Enzymatically treated samples were significantly
230 darker than breads without enzymatic treatment, and the darkness augmented with the
231 level of enzyme added. As it was expected, the enzyme level of amyloglucosidase
232 increased the release of glucose from the hydrolysis of amylose and amylopectin,
233 providing additional glucose that accelerates the Maillard reaction. In fact, Sharma &

234 Singh (2010) reported the use of amyloglucosidase to enhance bread crust colour.
235 Furthermore, colour of bread is an important quality associated with aroma, texture and
236 appearance features which are decisive for consumers.
237
238 No differences were detected in the water activity and moisture content of the crumb in
239 the different samples due to enzymatic treatments (results not showed). The enzymatic
240 treatment at levels higher than 100mg/10ml promoted a significant (P<0.05) decrease in
241 the crust water activity of the thick crust bread, and the reduction increased with the
242 level of enzyme. Considering that water activity refers to unbound or free water in a
243 system available to support biological and chemical reactions (Potter & Hotchkiss,
244 1998), it seems that the enzyme consumes molecules of water in the reaction of
245 hydrolysis of 1,4 and 1,6-α linkages of the starch, which reduces the amount of free
246 water in the bread crust. In the case of the thin crust bread, water activity showed a
247 decrease when amyloglucosidase was added up to 250mg/10ml, but the trend was
248 reversed when higher enzyme concentrations were added. A plausible explanation could
249 be that the enzyme penetrates the thin bread crust at high concentrations reaching the
250 bread crumb, which had significantly higher moisture content (40,9% in thick bread and
251 42.4% in thin bread) than the crust, facilitating the water molecules diffusion from the
252 crumb to the crust and leading an increase of the water activity.
253 Similar trend was observed when assessing the moisture content of the bread crust. The
254 enzymatic treatment produced a significant (P<0.05) decrease in the moisture content;
255 probably due to the participation of the water molecules in the hydrolysis reaction,
256 which led to drier crusts. Again, in thin crust bread the addition of up to 250mg/10ml
257 amyloglucosidase resulted in the lowest moisture content, which increased at higher
258 enzyme levels. In the thick crust bread, the effect was dependent on the enzyme level,

moisture content showed lower values with higher concentration. Therefore, greater enzyme levels were required for diffusing through the crust in breads with thicker crust. Water is the predominant constituent in most foods and it is a direct reactant in hydrolytic processes. Moreover, the change of cross link and entanglements between amylose and amylopectin caused by enzymatic treatment might increase the porosity, which favors the water release during the full baking yielding drier bread crust. According to Esveld et al. (2012), the moisture diffusion in cereal cellular products involves diffusive transport in the gas phase and in the solid phase, and both depend on the morphological details of the structure. Xiong et al. (1991) indicate that the mobility of water in solid foods is strongly dependent on the porosity of the structure. Porosity is intuitively related to macroscopic vapor transport rate while sorption rate in the solids seems more related to the local microscopic thickness of the solid (Esveld et al., 2012), which could explain the differences observed in the two specialties due to bread crust thickness.

A reduction in water activity and moisture content of the bread crust was previously observed by Altamirano-Fortoul & Rosell (2010) and Primo-Martin et al. (2006) when different enzymes were sprayed or added to study their effect in bread crust characteristics.

Mechanical properties

Altamirano-Fortoul et al. (2013) reported that the use of smaller punch cross section and low speed allow obtaining reliable information about the cellular structure of the bread crust. On the contrary, compression becomes more important with the use of greater punch cross section and high speed. According to the above, two sets of conditions (punch cross section of 3 mm^2 and 28 mm^2) were applied for determining the

284 mechanical properties of the crust to obtain information about the cellular structure and
285 the compression behavior. Table 3 shows the mean values obtained for the mechanical
286 parameters for each level of the factors (crust type, enzyme concentration, punch cross
287 section) and the statistical significance of each of the factors. It also shows the standard
288 error of each mean, which is a measure of its sampling variability. Regardless the punch
289 cross section used in the test, the enzymatic treatment produced significant changes on
290 all mechanical parameters used to define texture of the crust in the two bread specialties
291 studied (Table 3). The F*m* parameter was not significantly affected by the punch cross
292 section. Thick bread crust required greater force (F*m*) for breaking crust than thin crust.
293 The increase of enzyme level slightly reduced the puncturing force (*Fm*), and that effect
294 was more evident in thin bread crust, independently of the punch cross section and
295 speed (Figure 1). Thus, the amyloglucosidase was acting on the thin and thick bread
296 crust inducing changes at cellular structure level, leading fragile structure. In fact,
297 Luyten et al. (2004) describe that the force depends on the composition and the structure
298 of the food.
299 The reduction observed in the puncturing force due to enzyme action could be related to
300 the decrease in the moisture content and water activity. As mentioned above, water
301 leads to plasticization and softening of the starch-protein matrix and thus alters the
302 mechanical properties, and an increase in water content increases the response to force
303 (Jakubczyk et al., 2008). In the case of bread crust, Primo-Martin et al. (2009) described
304 that at a_w of 0.75 bread crust was fully plasticized, and the transition from glassy state to
305 rubbery state occurs at a_w of 0.68-0.69 leading an increase in the rupturing force
306 (Altamirano-Fortoul & Rosell, 2010).
307 Greater punch cross section (28 mm^2) and higher speed produced significantly
308 (P<0.001) less structural ruptures in all the samples (Table 3). Therefore, more

309 information about cellular structure was obtained at lower punch cross section and
310 slower speed. Spatial frequency of structural ruptures (N_{wr}) in the thick bread crust
311 showed significantly (P<0.001) higher values than thin bread crust (Table 3). In the case
312 of thick bread crust, at both punch cross sections and speeds, the number of structural
313 ruptures increased with the enzyme level up to 250mg/10ml amyloglucosidase, but
314 lower number of structural ruptures was observed at higher enzyme levels (Figure 2).
315 Considering that high number of fracture events is produced by crispy products, the
316 enzymatic treatment resulted in samples crispier than the control crust. Similar positive
317 effect was observed by Altamirano-Fortoul et al. (2013). Newly, these results might be
318 related to the decrease in water activity and an increase of the porosity due to the action
319 of the treatment. The decrease in water activity resulted in an increase of the jaggedness
320 of the force-displacement curve. Some authors reported that the increase of moisture
321 content or water activity of crispy food promote the loss in jaggedness of force-
322 displacement curve and consequently the frequency distribution of number of fracture
323 (Van Hecke, 1998; Jakubczyk et al., 2008; Tsukakoshi et al., 2008; Castro-Prada et al.,
324 2009; Arimi et al, 2010)
325 With respect to f_{wr} parameter, a significant (P<0.001) decrease was obtained with
326 enzyme treatment in both samples in comparison to their respective controls (Table 3).
327 Thick bread crust presented significantly higher values in this parameter than the thin
328 bread crust. Again, the effect of punch cross section showed an increase in f_{wr} parameter
329 when using 28 mm^2 compared with punch cross section of 3 mm^2. Considering f_{wr}
330 parameter relates the specific force with the structural ruptures, if treated bread crusts
331 required lower force to promote the fracture as well as showed greater number of
332 ruptures, it would be expected that this parameter will be lower than that in the control
333 bread crust. Amyloglucosidase sequentially detaches the glucose units allowing the

polysaccharide breakdown, which might have modified the cell wall associated to starch within the crust matrix. Consequently, the addition of enzyme reduced the mechanical resistance in the cell walls, leading to lower values of this parameter.

Recently, Altamirano-Fortoul et al. (2013) suggested that only by using low puncturing speed is possible to assess the crispness work, because of that it is only shown the values obtained by puncturing with small punch cross section and low speed (Figure 3). Crispness work parameter (W_c) showed a decrease with increasing the enzyme level in both bread specialties. Results obtained showed that with those puncturing settings was possible to detect the effect of enzymatic treatment on the mechanical behaviour of the crust. The observed effect could be related to the amyloglucosidase hydrolysis of long-chain polysaccharides causing an increase in the number of the pores, and in consequence less crispness work was needed. In fact, some authors reported that pores play a main role in the crispness and texture of foods (Goedeken & Tong, 1993; Tsukakoshi et al., 2008).

A multivariate analysis was applied to determine the possible correlation among the physicochemical properties and the parameters that defined the mechanical properties of the crust obtained with the 3 mm^2punch cross section. F*m* parameter showed a significant positive relationship with crispiness work (W_c) (r=0.9225), moisture content (r = 0.7041), and also significant but very weak correlation with water activity (r = 0.3145). A significant positive relationship was observed between the spatial frequency of structural ruptures (N_{wr}) and the total colour difference (r=0.6352). In addition, a significant positive relationship was obtained between the crispness work (W_c) and moisture content (r=0.7939).

358

359 Crust Structure

360 To achieve a better understanding of the enzyme action on the mechanical behaviour,
361 the microstructure of the crust cross-section was analyzed by SEM. The bread crust
362 with lowest and highest enzyme concentrations were selected for microstructure studies
363 with the purpose of observing the effect of the dosage of added enzyme.

364

365 Micrographs of control bread crusts and also those treated with amyloglucosidase
366 100mg/10ml (A1) and amyloglucosidase 1000mg/10ml (A4) are showed (Figure 5 and
367 6). Bread crusts with and without treatment revealed two different structural zones: a
368 dry crust and sub-crust. Similar structures were observed by Altamirano-Fortoul et al.
369 (2012), who suggested that the sub-crust is of great importance due to it lends rise to
370 chemical transition between the crust and the crumb, as well as this indicates where the
371 crust begins and ends. Figure 5a shows the thin bread crust without added enzyme
372 (control), where an uniform structure was presented, and at higher magnification
373 (Figure 6a) it was observed a smooth layer due to gelatinised starch, which covers quite
374 well the ungelatinized starch granules around the air cell. However, in untreated thick
375 bread crust (control) a cracking structure with a thicker zone 1 and bigger cells were
376 displayed (Figure 5b), besides starch granules could be clearly envisaged under a
377 smooth film showing little distorted structures (Figure 6b). Therefore, the distinct
378 mechanical properties observed in both specialties could be ascribed to their cellular
379 structure. Moreover, it is important to consider that the bread crust properties are
380 dependent on the breadmaking process and of many factors including for instance lower
381 water content, extent of heat and mass transfer at the bottom and top surfaces (Vanin &
382 Trystram, 2009).

383 Enzyme treatment modified the bread crust structure in both samples. Thin bread crust
384 treated with A1 revealed a more disordered structure with small irregular voids and
385 great cracks (Figure 5c). At higher magnification it seems that the starchy gel, that
386 initially covered the structure, was thinner, revealing underneath structures (Figure 6c).
387 In thin bread crust treated with A4, the structure was significantly different with
388 apparent compact structure but with sub-holes within the cells (Figure 5e), which agrees
389 with previous observations of Rojas et al (2000) when bread were formulated with alfa
390 amylase. Besides, the network was not continuous and sharper surface was detected
391 (Figure 6e), likely due to the hydrolysis of starchy compounds. Treatment A4 led to
392 bread crust with greater spatial frequency of small structural ruptures as result of a non-
393 homogeneous structure and the numerous sub-holes, which agrees with mechanical
394 results (N_{wr} parameter). These changes were mainly related to greater starch hydrolysis,
395 which altered the starch structure, resulting in a more porous structure. Therefore if the
396 microstructure is more porous, it gives brittle behavior (Goedeken & Tong, 1993).
397 In the case of thick bread crust treated with A1 an amorphous, disrupted and cracked
398 structure was observed (Figure 5d). Higher magnification allowed detecting some
399 deformed starch structure due to the partly disappearance of the covering layer, and
400 even some remnant intact starch granules (Figure 6d). In samples treated with A4 a
401 layered and fragmented structure was observed (Figure 5f). Likely, this different
402 structure might result from the intense hydrolysis through the crust that reach the
403 crumb, allowing some water molecules to diffuse and in consequence change the
404 structure. This different structure was confirmed at higher magnification (Figure 6f).
405 Moreover, these results agree with those observed in N_{wr} where different trend was
406 observed at higher enzyme activity. Most probably, enzyme level affected the protein-
407 starch interactions as well as the interaction between starch chains and water molecules,

and in consequence the granule's gelatinization. According to Guerrieri et al. (1997) certain proteins (purified gluten, gliadin and high molecular weight glutenin subunits) modified amyloglucosidase activity in model systems. The proteins had an effect on the starch hydrolysis, which is related to protein-starch interaction, especially when producing starch gelatinization. In addition, considering that the enzyme treatment reduced the amount of water available in the bread crust, starch gelatinization would be rather limited. Altamirano-Fortoul et al. (2012) found that lower amount of water present in the bread crust limited the gelatinization, which yield a more porous network with intact granules and partially gelatinized starch granules. Consequently, those effects can be related with the formation of successive structure layers (sandwich-like structures) in the sample treated with A4. The sample composed of long cell walls disrupted more easily when performing the fracture, resulting in lower values in the puncturing force parameter as were detected when texture was determined with small puncturing at low punching speed. Stokes & Donald (2000) indicated that when starch and gluten matrix are in a glassy state cell walls become more prone to fracture.

In general, the effect of the enzyme on microstructure of bread crust was dependent on enzyme dosage and the type of bread crust (thin or thick). Amyloglucosidase action resulted in a more disrupted structure with partly removal of the gelled film that covered the starch granules. Previous studies showed that enzyme treatment modified the morphology and characteristics of bread crust (Primo-Martin et al., 2006; Altamirano-Fortoul & Rosell, 2010). Therefore, it is of special interest to know the microstructure of the bread crust because it is responsible for the puncturing behavior.

4) Conclusion

Present study shows that enzymatic treatment of the bread crust decreased the moisture content and water activity, due to an increase in the crust porosity besides the removal of water participating in the hydrolysis reaction. Enzyme addition affected the colour crust; in general an increase in the total colour difference was observed when enhancing the enzyme concentration. Regarding mechanical properties, overall results indicate that the enzymatic treatment resulted in crust with reduced resistance to puncture and high number of fracture events, indicating crispy products. In addition, crispness work parameter was lower as consequence of the fragility of the crust. The correlation matrix revealed the positive relationship of the moisture content with F*m* and W_c when studying the effect of amyloglucosidase on the crust.

Furthermore, the results of the SEM analysis also confirmed the effect of the enzymatic treatment. Amyloglucosidase hydrolyzed the starchy gel of the crust exposing the starch granules and resulting in a more irregular and uneven structure. This study suggest that the enzyme produced an important modification on the starch-protein matrix structure, related to the steady removal of the gelatinized starchy layer that cemented the matrix, which validate the results on the physicochemical and puncturing parameters. The enzyme level required for modulating crust structure was dependent on the crust thickness.

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FIGURE CAPTIONS

FIGURE 1. Effect of enzyme treatment on the puncturing force (*Fm*) of thin (closed symbols, ●, ▼) and thick (open symbols, o,▽) bread crust. Legends: 3 mm^2 punch cross section at 0.5 mm/s (●), 28 mm^2 punch cross section at 40 mm/s (▼).

FIGURE 2. Effect of enzyme treatment on the frequency of structural ruptures (N_{wr}) of thin (closed symbols, ●, ▼) and thick (open symbols, o,▽) bread crust. Legends: 3 mm^2 punch cross section at 0.5 mm/s (●), 28 mm^2 punch cross section at 40 mm/s (▼).

FIGURE 3. Effect of enzyme treatment on the crispness work (W_c) of thin (closed symbols, ●) and thick (open symbols, o) crust breads.

FIGURE 4. Scanning electron micrographs of crust cross section. Magnification of 50x. Images correspond to cross section of breads with thin (a, c, e) and thick (b, d, f) crusts. Micrographs of control crust (a, b), crust treated with amyloglucosidase 100mg/10ml (c,d) and amyloglucosidase 1000mg/10ml (e, f**).**

FIGURE 6. Scanning electron micrographs of crust cross section at high (1500x) magnification. Images correspond to cross section of breads with thin (a, c, e) and thick (b, d, f) crusts. Micrographs of control crust (a, b), crust treated with amyloglucosidase 100mg/10ml (c, d) and amyloglucosidase 1000mg/10ml (e, f**).**

592 Table1. Enzyme concentrations applied onto the bread surface (2 ml were applied per

593 loaf).

594

Treatment	Code	Description	Dosage 595
Control	C	Distilled water	0mg/10 ml
AMG	A1	Amyloglucosidase	100mg/10ml
	A2	Amyloglucosidase	250mg/10ml
	A3	Amyloglucosidase	500mg/10ml
	A4	Amyloglucosidase	1000mg/10ml

597 Table 2. Effect of amyloglucosidase on the physicochemical properties of thin and thick bread crust.

Bread crust	Enzyme concentration (mg/10ml)	Aw crust	Moisture content (%)	*L**	*a**	*b**	Δ*E*	Darkness crust
Thin	0	0.516 ±0.02 c	9.67 ±0.10 g	54.26 ±0.53 e	14.87 ±0.40 e	37.42 ±0.56 c	0 ±0 a	45.74 ±0.53 b
	100	0.498 ±0.01 bc	6.22 ±0.12 d	55.12 ±1.77 e	14.09 ±0.90 de	36.49 ±1.03 c	2.65 ±0.58 b	44.88 ±0.77 b
	250	0.481 ±0.01 b	5.43 ±0.32 c	48.44 ±0.40 c	12.72 ±0.01 ab	20.53 ±0.68 a	18.10 ±1.15 e	51.56 ±0.04 d
	500	0.552 ±0.03 d	6.73 ±0.08 d	49.22 ±0.70 cd	13.96 ±0.23 d	27.29 ±0.55 b	11.44 ±0.68 c	50.78 ±0.70 cd
	1000	0.505 ±0.01 c	8.02 ±0.10 f	44.86 ±0.59 a	13.70 ±0.69 cd	27.04 ±0.48 b	14.06 ±0.86 d	55.14 ±0.59 f
Thick	0	0.540 ±0.00 d	11.46 ±0.22 h	60.82 ±0.24 f	11.85 ±0.45 a	35.21 ±0.46 c	0 ±0 a	39.18 ±0.25 a
	100	0.540 ±0.04 d	6.10 ±0.05 d	49.98 ±0.21 d	12.42 ±0.21 ab	20.82 ±0.75 a	18.06 ±1.01 e	50.02 ±0.21 c
	250	0.507 ±0.01 c	5.27 ±0.15 b	48.51 ±0.55 c	12.99 ±0.83 bc	20.97 ±0.87 a	18.86 ±0.83 ef	51.49 ±0.56 d
	500	0.459 ±0.01 a	5.30 ±0.03 bc	46.74 ±0.08 b	12.92 ±0.53 bc	21.80 ±0.36 a	19.50 ±0.84 f	53.26 ±0.26 e
	1000	0.452 ±0.01 a	4.93 ±0.02 a	45.85 ±0.12 ab	12.40 ±0.74 ab	23.39 ±0.79 a	19.18 ±0.78 f	54.15 ±0.13 ef

598 Means and standard deviations sharing the same letter within a column were not significantly different ($P < 0.05$).

599 **Table 3.** Effect of enzyme level and punch cross section on puncturing parameters in

600 two different bread specialties.

Factor	F*m* (N)		N_{wr} (m^{-1})		f_{wr} (N)	
	Mean	SE	Mean	SE	Mean	SE
GRAND MEAN	7.53		1.57		12.04	
Bread crust	***		***		***	
Thick	9.82	± 0.71	2.36	± 0.16	13.59	± 0.42
Thin	5.24	± 0.71	0.79	± 0.16	10.50	± 0.42
Enzyme concentration (mg/10ml)	***		***		***	
0	11.94	± 1.13	0.71	± 0.26	20.31	± 0.67
100	6.78	± 1.13	1.94	± 0.26	11.46	± 0.67
250	6.30	± 1.13	2.17	± 0.26	9.96	± 0.67
500	6.51	± 1.13	1.20	± 0.26	9.81	± 0.67
1000	6.10	± 1.13	1.85	± 0.26	8.68	± 0.67
Punch cross section (mm^2)			***		***	
3	6.57	± 0.71	2.76	± 0.16	6.22	± 0.42
28	8.48	± 0.71	0.39	± 0.16	17.87	± 0.42

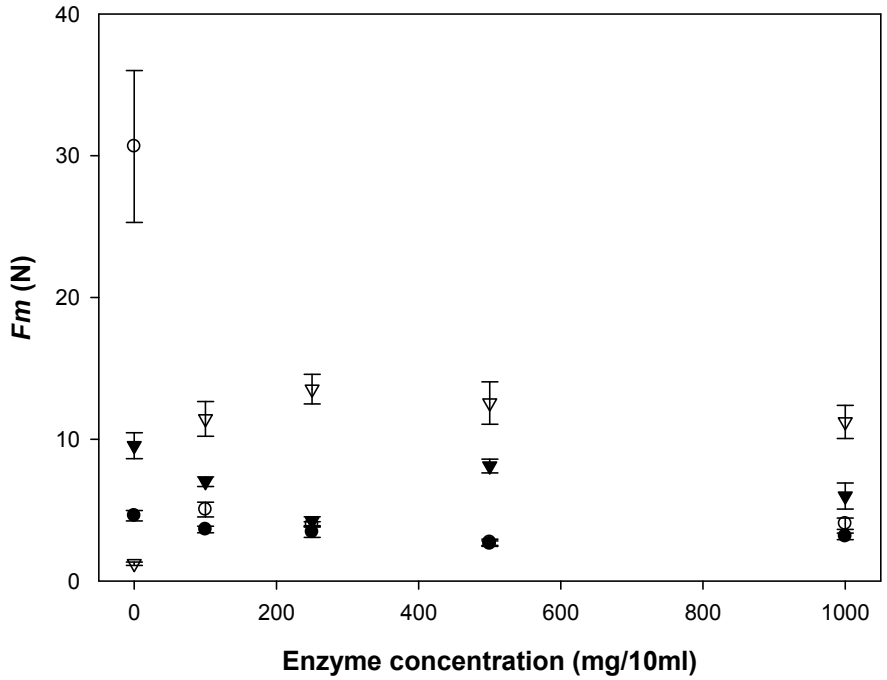
601 Means values + standard error (SE). The standard error of each mean is a measure of its

602 sampling variability.

603 * Significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$.

604

605 Figure 1.



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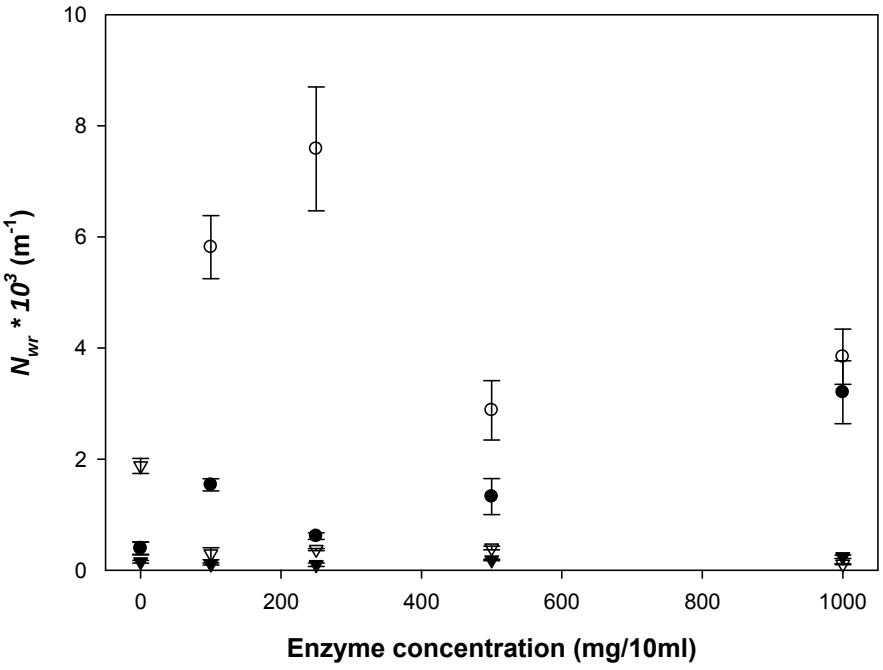
608 Figure 2.



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610 Figure 3.

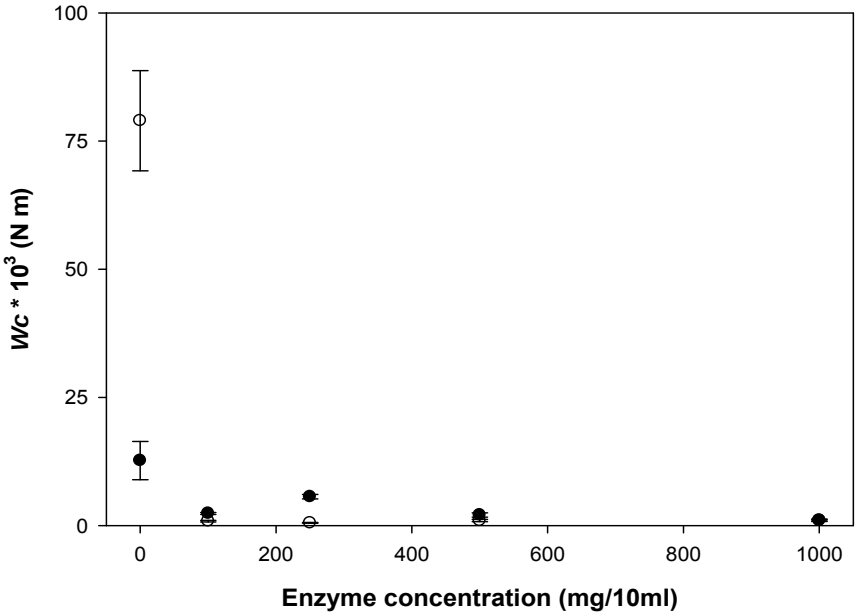

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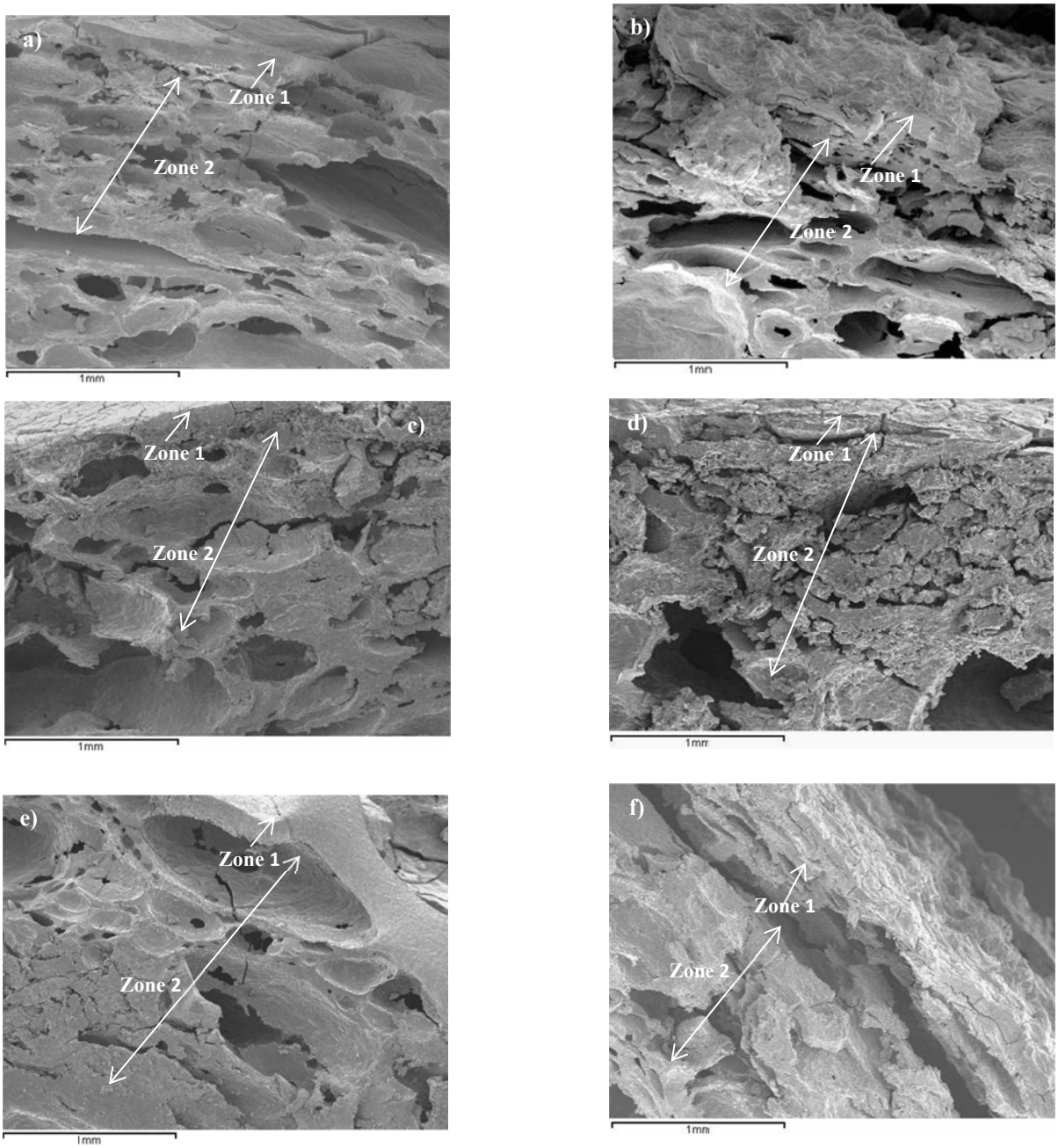
Figure 4


a)
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b)
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637 Figure 5

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