**Modulation of in vitro digestibility and physical characteristics of
protein enriched gluten free breads by defining hydration**

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Abstract

The association of diabetes of celiac disease together with the high glycemic index of gluten-free breads makes necessary the pursuit of a solution to reduce this glycemic response. Protein enrichment and changes on hydration level may be tools to get it. Thus, the aim of this study was to analyze the effect of two proteins (pea and egg white) on the digestibility and physical characteristics of gluten-free breads, with either the same hydration or specific volume. Protein enriched breads were made blending starch and proteins (70:30). Breads prepared at constant hydration presented lower specific volume and weight loss during baking, and higher hardness than control. However, when the specific volume was matched, the breads with egg white protein presented lower weight loss and higher hardness, while the ones with pea protein were the less hard. Protein enriched breads showed higher SDS and lower RDS values than control with constant hydration, but lower SDS values and glucose release with constant specific volume. Therefore, the bread volume influenced not only the physical properties but also the bread digestibility.

1. Introduction

Consumption of gluten-free products has been increased over last years, partly due to greater number of celiac patients diagnosed, which has to adhere to a gluten-free diet lifelong, but also due to a growing number of healthy followers of a gluten-free diet, since they assume to be healthier. This restrictive diet forces to counterpart products, which do not include gluten among their ingredients. Nevertheless, all gluten-free products available in the market present a shared problem, their poor nutritional composition: low complex carbohydrates, protein, minerals and vitamins contents (Matos & Rosell, 2015).

Most of gluten-free breads (GFB) contain mainly starches and refined flours from gluten-free raw materials and show higher glycemic response than normal white breads (Fardet, Leenhardt, Lioger, Scalbert, & Rémésy, 2006). Glycemic response depends on indigenous factors (granule structure, protein and lipid content, amylose:amylopectin ratio, digestion conditions and particle size) and the macroscopic structure of the food (de la Hera, Rosell, & Gómez 2014; Wolter, Hager, Zannini, & Arendt, 2013). Some authors reported that the presence of protein can decrease glycemic response due to the interactions with starch (Fardet et al., 2006). Therefore, the addition of proteins not only would improve the nutritional composition of GFB, but also would help to maintain a good glycaemic control. In addition to all above, it is important to underline that as well as of gluten-free products, an increasing demand of protein enriched products has also been observed over last years (Banovic et al., 2018). In this way, the addition of exogenous proteins also allows to obtain products with high protein content and to cover the market demand of this type of products. Several studies have been focussed on evaluating the glycaemic index of GFB (de la Hera et al., 2014; Matos & Rosell,

2011; Wolter et al., 2013). However, none of prior studies assessed the effect of protein enrichment on that glycaemic index.

In GFB, hydration of ingredients significantly affects the rheological properties of doughs and bread characteristics, leading to higher volume (Sahagún & Gómez, 2018). In addition, it has been reported the influence of hydration level of doughs on GFB starch digestibility (de la Hera et al., 2014).

The aim of this study was to determine the impact of protein enrichment and hydration level on the GFB quality parameters and *in vitro* digestibility of proteins and starch. To assess how the protein enrichment and the hydration level influence the final product, GFB with a substitution of 30% of starch by pea or egg white protein were elaborated at either constant hydration level or optimum hydration level to obtain constant specific volume (5.5 ± 0.1 mL/g).

2. Materials and methods

2.1. Materials

Maize starch (7.83% moisture, 0.76% water binding capacity; Tereos, Zaragoza, Spain), pea protein isolate (Nutralys F85 M; 78.13% protein, 6.16% moisture, 5.40% water binding capacity; Roquette, Lesterm, France) and egg white protein powder (81.66% protein, 6.18% moisture, 0.00% water binding capacity; EPS S.P.A., Occhiobello, Italy) were used. Moisture and water binding capacity were measured following AACC methods 44-16.01 and 56.30, respectively (AACC International, 2012). Other ingredients used for bread making were sugar (AB Azucarera Iberica, Valladolid, Spain), refined sunflower oil (Langosta, F. Faiges, S.L., Daimiel, Ciudad Real, Spain), salt (Disal, Unión Salinera de España S.A, Madrid, Spain), hydroxypropyl

79 methylcellulose K4M (Rettenmaier & Sohne, Rosenberg, Germany), and instant dry
80 baker's yeast (Dosu Maya Mayacilik A.Ş, Istanbul, Turkey).

81 *2.2. Bread making*

82 The recipe used in bread making (g/100 g of starch) were: maize starch (100 g), oil (6
83 g), sugar (5 g), yeast (3 g), HPMC (2 g) and salt (1.8 g). For enriched samples, 30% of
84 starch was substituted by pea (P) or egg white (EW) proteins. Breads were made
85 applying constant hydration (CHL) or adapted hydration to obtain targeted specific
86 bread volume (CL) (5.5 ± 0.1 ml/g). For constant hydration 90%, starch basis, was used.
87 Based on preliminary trials, the hydration level (% starch basis) required for each
88 starch-protein mixture to obtain a specific volume of 5.5 ± 0.1 mL/g was established:
89 control 65%, pea protein 133% and egg white protein 69%.

90 First of all, the instant yeast was rehydrated before using. Next, the rest of ingredients,
91 except yeast and water, were mixed at speed 1 for 1 min using a KitchenAid
92 Professional mixer (Kitchen Aid, St. Joseph, Michigan, USA). The rehydrated yeast
93 was incorporated to the previous mixture and mixed at speed 2 for 8 min. Once
94 obtaining a homogenous blend, 150 g of dough pieces were placed into oil-coated
95 aluminum pans ($159 \times 109 \times 39$ mm) and fermented at 90% RH and 30 °C for 60 min.
96 After fermentation, the doughs were baked at 190 °C for 40 min. Finally, loaves were
97 removed from the pan, left to cool for 1 h at room temperature and packaged in
98 polyethylene bags. Breads were stored at 24 °C till further analysis. Two batches of
99 breads were performed for each recipe.

100 *2.3. Physical bread characteristics*

101 Specific volume is defined as the ratio between the volume of a piece and its weight.
102 Bread volume was measured using a laser-based scanner (Volscan Profiler; Stable

Microsystems, Surrey, UK) and conducted on three loaves from each elaboration.

Weight loss of breads was calculated based on the following formula:

$$\text{Weight loss (\%)} = \frac{\text{Batter weight (150 g)} - \text{Bread weight}}{\text{Batter weight (150 g)}} \times 100$$

Both parameters were measured of three breads from each repetition one day after baking.

Crumb texture was determined using a TA-XT2 texture analyser (Stable Microsystems, Surrey, UK). A 25-mm diameter cylindrical aluminium probe was used in a “Texture Profile Analysis” (TPA) double compression test to penetrate to 50% of the depth at a test speed of 1 mm/s with a trigger force of 5 g and a 10-sec delay between the first and second compressions. Texture measurements were performed on two central slices (20 mm thickness) of two loaves from each repetition (2x2x2). Hardness (N), springiness, cohesiveness, chewiness and resilience were calculated from the texture profile analysis graph. Δ Hardness (%) was calculated as the percentage of the ratio between the hardness of breads at day 1 and day 5 after baking.

2.4. In vitro starch digestibility and estimated glycemic index

Protein enriched GFB were frozen, freeze-dried and ground in a blender. Then, *in vitro* starch digestibility was determined in the powders following the method described by Benavent-Gil and Rosell (2017). Glucose was determined using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). Starch was calculated as $\text{glucose (mg)} \times 0.9$. Breads subjected to *in vitro* digestion in the absence of protein were employed as controls. According to the hydrolysis rate of starch, three different fractions were quantified and expressed as the amount of glucose released (mg/100 mg) (Englyst, Veenstra, & Hudson, 1996). Rapidly digestible starch (RDS) was defined as the starch fraction that was hydrolyzed within 30 min of incubation, slowly digestible starch (SDS) was the starch fraction hydrolyzed within 30 and 120 min, and resistant

starch (RS) was defined as the starch fraction that remaining unhydrolyzed after 16 h of incubation.

The experimental data were fitted to a first-order equation to calculate the kinetics of *in vitro* digestion (Goñi, Garcia-Alonso, & Saura-Calixto, 1997): $C = C_{\infty} (1 - e^{-kt})$ where C was the concentration at t time, C_{∞} was the equilibrium concentration or maximum hydrolysis extent, k was the kinetic constant and t was the time chosen. The hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve (0–180 min) of the sample by the area of a standard material (white bread) over the same period of time. The expected glycemic index (*eGI*) was calculated using the equation $eGI = 8.198 + 0.862HI$ (Grandfeldt, Bjorck, Drews, & Tovar, 1992).

2.5. The *in vitro* protein digestibility

Digestibility of protein in control and protein enriched samples was determined according to the *in vitro* method described by Espinosa-Ramírez, Garzon, Serna-Saldivar and Rosell (2018). Previously, the protein content was quantified following Dumas Combustion Principle using 6.25 as conversion factor. The percent of protein digestibility (Y) was quantified by using the equation $Y = 210.464 - 18.1x$ where x is the change in pH after 10 min (Hsu, Vavak, Satterlee, & Miller, 1977).

2.6. Statistical analysis

All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean $\pm$ standard deviation. Fisher's least significant differences test was used for assessment of significant differences among experimental mean values with 95% confidence. Pearson correlation coefficient (r) and P -value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). Differences of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Specific volume and weight loss of breads

The effect of bread making process and protein source on the specific volume and weight loss of breads is shown in Table 1. When the hydration level was constant (CHL), the incorporation of protein reduced significantly the specific volume, especially when P protein was included, as Sahagún and Gómez (2018) already observed. To obtain breads with the same specific volume (CV), those containing P protein required a higher hydration level, as Sahagún and Gómez (2018) reported previously, due to its high water binding capacity. In this same study, it was observed that the influence of hydration level on the volume of breads with EW protein was less dramatic than the control or P breads, and that for the volume selected the hydration required was similar than control, despite their lower water binding capacity.

Regarding weight loss of breads with CHL, the control sample, which showed the highest volume, also presented the greatest weight loss. Large bread surface might increase the air exchange surface of the breads, which agree with reported findings (de la Hera et al., 2014). However, due to their higher specific volume, it would be expected that the breads with EW protein show higher weight loss than the ones with P protein, but, no significant differences were observed between both samples. In fact, among the breads with a CV, control and P breads did not show significant differences between them despite the vast differences on hydration level, while the EW breads presented lower weight loss values. P breads were made with higher amount of water than control samples, so it could be expected higher weight loss. However, the protein presented great water binding capacity and it is able to retain it after baking. In the case of breads with EW protein, as it was observed with a CHL, the incorporation of this protein reduced the weight loss during baking of breads with a CV. This fact could be explained

by the compact structure of these breads (Figure 1) with a uniform bubble distribution, since the water diffusivity depends on porosity and average pore size (Karathanos & Saravacos, 1993). Moreover, it is important to keep in mind that, although the water binding capacity of EW protein is much lower than starch, when this protein is heated and experience the coagulation process, the water binding capacity of both ingredients is similar (Bravo-Núñez and Gómez, 2019).

3.2. Textural characteristics of breads

Texture parameters of breads were evaluated, with exception of P bread with CHL, whose low specific volume did not allow it (Table 2). It is widely accepted the negative relationship between specific volume and hardness. Nevertheless, breads containing EW protein, despite their lower specific volume compared to the control, had harder crumbs independently on the amount of water used for bread making (CV or CHL). Same results have been previously observed and attributed to the coagulation process of the egg protein during baking (Kiosseoglou & Paraskevopoulou, 2006). Concretely, the low endothermic enthalpy of albumen allows its unfolding in the early stages of baking (Nozawa, Ito, & Arai, 2016), facilitating the formation of disulphide bridges among the albumen molecules giving rise to a strong and elastic gel network structure (Kiosseoglou & Paraskevopoulou, 2006). In consequence, the structure of the bread crumbs was different (Figure 1). Breads containing EW protein presented a compact alveolar structure with great number of small air cells. Conversely, for the same specific volume, P-CV bread showed less dense structure leading to the softest crumb. Regarding the hardness after storage, it was almost twice after 5 days' storage. But when the hardness increase was compared, there were not significant differences among the breads, with the exception of the bread containing EW and made using CHL, which showed softer crumbs during longer period, despite its initial harder crumb.

Regarding the rest of texture parameters, the bread making process and type of protein did not affect springiness and chewiness followed the same trend than hardness, since it is a secondary parameter dependent on hardness. The addition of EW protein led to high cohesiveness compared to the control, particularly significant with CV, in agreement with Sahagún and Gómez (2018), likely due to the thermal coagulation and elastic gel formation, previously mentioned.

3.3. Determination of protein digestibility

Protein enrichment affected in different extent the protein content and the effect was dependent on the protein type and the bread making process. The protein content in control samples (CV and CHL) averaged 1.81 ± 0.08 g/100 g dry matter, which is comparable to that of commercial GFB (Matos & Rosell, 2011). Taking into account that control bread was made solely of maize starch, the protein content might come from the yeasts. As expected, the protein enriched samples contained significantly higher percentage of protein (23.57 g/100 g dry matter and 23.93 g/100 g dry matter when containing EW or P proteins, respectively) than control samples. Although proteins content apparently decreased after the bread making process, since doughs were enriched with higher levels of protein (30 g / 100 dry matter in starch) than those observed in their corresponding breads. This agrees with previous reports in which the protein content of bread elaborated with spelt and wheat flours decreases after mixing and proofing (Abdel-Aal, 2008). The different hydration tested in both bread making processes did not induce significant differences.

Protein digestibility as a measure of the susceptibility of a protein to proteolysis (Hsu et al., 1977) was evaluated in all the breads by measuring the pH (Figure 2). The statistical analysis revealed that the protein enrichment significantly ($P < 0.05$) modified the *in vitro* protein digestibility, while bread making process did not prompt significant effect

on this parameter. Regardless bread making process, the *in vitro* protein digestibility in control samples ($69.24 \pm 0.58 \%$), made only with maize starch, was the lowest among samples studied. The value was considerably lower than that obtained for rice based GFB ($78.15 \pm 0.36 \%$) (Espinosa-Ramírez et al., 2018). Therefore, GFB made with maize starch had very low amount of protein with lower digestibility. The protein enriched breads showed a rapid pH drop compared to control breads (Figure 2). Breads containing EW and made reaching constant specific volume (CV) had significant lower digestibility ($76.53 \pm 1.30\%$) than the rest of the enriched breads ($79.03 \pm 0.30\%$). Higher values of *in vitro* protein digestibility have been associated to lower interaction between proteins and starch, making them more accessible to proteases (Hsu et al., 1977). According to this, EW proteins might have stronger interaction with maize starch than P proteins, hindering the protein hydrolysis and thus reducing the pH decline. The pH decline was similar in the other protein enriched breads indicating similar accessibility to proteases.

3.4. Starch fractions of different digestibility in enriched GF breads

From the nutritional point of view, the starch can be fractionated into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) according to its rate and extent of *in vitro* digestion. RDS and SDS fractions have been related to the rate at which glucose is available for absorption in the small intestine (Englyst et al., 1996), while RS fraction is not absorbed in the small intestine of healthy individuals (Skrabanja, Laerke, & Kreft, 1998). The possible influence of protein type and bread making process was evaluated in the EW and P protein enriched GFB using different baking processes (CV or CHL).

The predominant starch fraction in control breads was SDS followed by RDS and RS (Figure 3a). SDS fraction is more desirable than RDS, offering the advantage of being

slowly digested in the small intestine and inducing gradual increase of postprandial plasma glucose and insulin levels (Jenkins et al., 1978). This pattern did not change when GFB were enriched with EW or P protein. But the amount of RDS and SDS fractions was greatly dependent on the bread making process, particularly the SDS fraction, which agrees with previous report (Lau, Soong, Zhou, & Henry, 2015). Higher values of those fractions were expected when higher hydration was used because it would allow extended starch gelatinization. Nevertheless, despite the high hydration level of P-CV (133%, starch basis), no significant differences were found on the RDS and SDS levels compared to EW-CV; thus, starch gelatinization plus microstructural interactions within bread constituents might affect enzymes accessibility and the subsequent starch hydrolysis. In general, enriched samples displayed a significant reduction in the RDS fraction, except in the case of P protein that did not modify this fraction in GFB with CV. Overall, protein enriched GFB with CHL were found to contain significantly lower amounts of RDS, with a concomitant increase in the SDS, suggesting that SDS resulted from the transformation of RDS fraction (Figure 3a). Therefore, EW reduced RDS both with CV and CHL, presumably starch-protein interactions hindered the starch hydrolysis. The SDS fraction in GFB decreased when protein enriched samples were baked with CV. Meanwhile, protein enriched samples with CHL increased this fraction compared to their respective control. Further, as it has been explained previously, lower amount of accessible water in the system may be related to a reduced gelatinization due to limited dough moisture. Therefore, the incorporation of protein into the GF blends may have increased SDS levels by reducing the activity and accessibility of the *in vitro* digestive enzymes as a result of not fully starch gelatinization and a reduction of bread specific volume (Table 1) (Cavallero, Empilli, Brighenti, & Stanca, 2002).

Regarding the RS fraction, different patterns were observed without no clear trend. EW protein enrichment did not modify the RS content in GFB with CV, whereas P protein enrichment decreased this fraction. Conversely, the addition of egg protein increased RS content in GFB with CHL, while P protein addition did not significantly modify the RS content.

3.5. Hydrolysis kinetics and estimated glycaemic index

Figure 3b illustrates the hydrolysis plots resulting from the *in vitro* digestion of protein enriched GFB using different bread making process (CV or CHL). Hydrolysis plots were obtained by quantifying the release of glucose at specific intervals during the reaction. In general, all bread samples displayed a similar digestion behaviour in which the amount of glucose exhibited a linear increase at the early stage of hydrolysis (30 min), and which progressively decreased as hydrolysis proceeded (90-120 min). Similar hydrolysis behaviour has been previously reported in studies of the digestibility of commercial GFB (Matos & Rosell, 2011).

The influence of the protein enrichment and bread making process was evaluated on primary and secondary parameters derived from the *in vitro* digestion of GFB. These parameters are summarized in Table 3, including kinetics constant (k), equilibrium concentration of hydrolysed starch (C_{∞}), area under the hydrolysis curve after 180 min (AUC 180), hydrolysis index (HI) and estimated glycaemic index (eGI).

The kinetics constant (k), which indicates the hydrolysis rate in the early stage, was not significantly modified by any of the factors studied. Nevertheless, as observed in the hydrolysis plots (Figure 3b), protein enriched breads made at CHL were slowly hydrolysed (lower k) during the early stage of digestion, likely due to the lower amount of RDS fraction (Chung, Shin, & Lim, 2008). The maximum hydrolysis, C_{∞} , was significantly influence by bread making process. All protein enriched samples with CV

showed lower C_{∞} values than their respective control, but no significant differences were observed in samples with CHL. Previous studies have also demonstrated that high-protein starchy foods displayed reduced digestion rate compared with low-protein starchy foods (Jenkins, Thorne, Wolever, Rao, & Thompson, 1987). The decrease in digestibility may be explained by the starch–protein interaction, which extends the starch digestibility during enzymatic hydrolysis (Guha, Ali, & Bhattacharya, 1997). Some studies suggested that the interaction between starch and protein is composed of granules with a starch core surrounded by a protein network (Gordon, Davis, & Timms, 1979). The protein network might result in a significant reduction of the digestible enzyme accessibility by blocking the adsorption sites and thus causes a lower binding of the enzyme (Oates, 1997). Nevertheless, the results obtained suggested that bread making process plays an important role in the digestion rate. Regardless breadmaking process and type of protein, all enriched samples displayed lower HI and AUC. In consequence, the protein enrichment lowered the *eGI* of the resulting GFB. Therefore, the addition of P protein as well as EW protein in GF doughs allow obtaining GFB with higher protein content and lower glycaemic index.

4. Conclusions

Protein enrichment of GFB induced a significant decrease in the specific volume, resulting in harder crumbs, particularly when high levels of water are required to hydrate the proteins. However, optimizing the level of water added for bread making, high specific volumes could be obtained independently of the protein type used for enrichment. Regarding *in vitro* digestibility, protein enrichment induced a significant increase in the *in vitro* protein digestibility values. The incorporation of proteins reduced the RDS fraction except to the pea protein with constant volume, which was not modified because of an excess of water. SDS decreased when hydration was optimized

to reach constant bread volume and in opposition, SDS increased when proteins were added using constant hydration in bread making. Finally, it is possible to conclude that the addition of pea protein as well as egg white protein in GF doughs allow obtaining GFB with higher protein content and *in vitro* protein digestibility, and a lower glycaemic index.

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