Article type: general paper

Title: IMPACT OF α-AMYLASE DURING BREADMAKING ON *IN VITRO* KINETICS OF STARCH HYDROLYSIS AND GLYCAEMIC INDEX OF ENRICHED BREAD WITH BRAN

Running title: GLYCAEMIC INDEX OF BREAD SUPPLEMENTED WITH α-AMYLASE AND BRAN

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Abstract

Nowadays, the use of enzymes has become a common practice in the bakery industry, as they can improve dough quality and texture of final product. However, the use of *α*-amylases could have a negative effect in the glycaemic load of product, due to the released sugars from the starch hydrolysis that are not used by yeasts during the fermentation process. This study evaluated the effect of the addition of α-amylase in bakery products with bran on *in vitro* kinetics of starch hydrolysis. The use of flour with a high degree of extraction or high bran amount could decrease the GI even with the

inclusion of α-amylase in the formulation. It should be taken into account the amount of bran and α-amylase when formulating breads in order to obtain products with lower GI than white bread. However, the fact that kinetics of starch hydrolysis remained unaltered indicates that the use of α-amylase in bread-making processes could provide technological advantages improving quality of breads without markedly changes in their glycaemic index.

Key Words: bread, glycaemic index, α-amylase, wheat bran, bran particle size distribution, kinetics of starch hydrolysis

Abbreviations: Am, fungal α-amylase addition; AUC, area under the curve; EC, enzyme commission number; GI, glycaemic index; La, large size; SD, standard deviation; Sm, small size; T2D, type 2 diabetes; TSH_{90}, total starch hydrolysed at 90 min; WB, white bread; 10, 10% of bran; 20, 20% of bran

Introduction

The glycaemic index (GI) has been proposed as a ranking based on the blood glucose response after eating a food, relative to consumption of white bread or a glucose solution [1]. Over the past years, studies regarding nutrition reveal that the optimal diet includes low GI foods. Scientific evidence has shown that high-GI carbohydrates are associated with increased risk of metabolic disorders such as obesity and type 2 diabetes (T2D) affecting large groups of population worldwide [2]. Accordingly, individuals who followed a low-GI diet over many years were at a significantly lower risk for developing T2D and co-morbidities such as coronary heart disease, insulin-resistance syndrome and some types of cancer [3-5].

Some strategies for reducing the glycaemic response in bakery products are the use of whole grains as well as the addition of external parts of the kernel, sourdough fermentation and/or addition of organic acids, or using cereal genotypes with high contents of amylose or β-glucans [3]. Whole-wheat or bran enriched breads contribute to a healthy dietary profile because of their higher content of complex carbohydrates, minerals, vitamins, antioxidants, and other biologically active phytochemicals. Dietary fibre plays an important role in the slow-release of glucose [3], therefore the products which containing it generally display lower GI than their fibre-free counterparts, maintaining better control of blood sugar level [6]. However, the addition of bran or whole flours affects negatively the final bread performance. Bran supplementation usually weakens the crumb structure, affects negatively its elasticity and decreases the loaf specific volume [7-8]. These effects have been attributed to the dilution and/or disturbance of the gluten network, which affect negatively the gas-holding capacity of dough [7, 9-10].

Enzymes are key tools within the modern bakery, since they provide higher quality to baked products. Enzymes have a proven track record in baking by providing improved dough handling and process tolerance, increased specific volume, finer crumb structure and softer crumb, besides extended shelf life [11-16]. Consequently, the importance of enzymes is likely to increase as consumers demand more natural products free of chemical additives. Some examples are cellulases, hemicellulases, pentosanases, proteases, lipases and oxidases, while by far the most used are the α-amylases. The α-amylases randomly hydrolyze α-1,4 glucosidic linkages in polysaccharides, resulting in short chains further fermented by yeast. The activity of α-amylases in dough systems and during baking impacts several product characteristics, including bread volume, firmness, and shelf life [11- 12]. However, these released saccharides obtained from the hydrolytic

activity of α-amylases, and not utilized by yeasts, may affect negatively the glycaemic response in the organism.

A high correlation has been found between the release rate of glucose in starchy foods by methods using digestive enzymes *in vitro* and *in vivo* glycaemic response [4]. Due to the high complexity and cost of GI evaluation in humans, the use of *in vitro* methods that mimic the human digestive process constitutes an useful tool predicting the glycaemic response after food intake [17].

The objective of this investigation was to assess the effect of the addition of α-amylase in bread formulations with bran on *in vitro* rate of starch digestion. Special attention was given to bran particle size and its influence on GI.

Materials and Methods

Materials and reagents

The characteristics of the commercial wheat used in this study were: moisture 11.23±0.03%, protein (N × 5.7) 11.11±0.05%; lipid content 1.81±0.02%, and ash 1.51±0.01%. Compressed yeast was used as a starter for bread making process. Fungal commercial α-amylase (EC 3.2.1.1) used in this study was from *Aspergillus oryzae* (Fungamyl BG, Novozymes - Bioindustrial, Madrid, Spain). Digestive enzymes were purchased from Sigma Chemical (St Louis, MO, USA): pepsin [EC 232-629-3] (Sigma, P7000), α-amylase (Sigma, A6255) and amyloglucosidase (Sigma, 10115). Working solutions of enzymes were prepared immediately before use.

Milling and bread making procedure

After appropriate cleaning, 600 g of wheat was tempered adding the adequate amount of water to 15.5% moisture in a Chopin Conditioner. The tempering was carried out at

20ºC during 16 hours. Milling test was performed on a Chopin Laboratory Mill (Tripette et Renaud, France). The wheat flour and bran obtained after milling was used into bread dough formulation. To obtain the smaller bran particle size, it has been ground in laboratory mill (Nanlysenmühle A10, Janke & Kunkel, Germany). Particle size distribution of the bran before and after grinding was determined by using a set of standard sieves (CISA, Barcelona, Spain) [7].

The bread dough formula (300g) consisted of wheat flour with 10 or 20% of bran at two different particle size distribution (corresponding to average diameters: 800 μm (Large size, La) and 300 μm (Small size, Sm), respectively), with or without the addition of α-amylase (0.5 U kg^{-1} of flour), compressed yeast (3.0 % flour basis), salt (2.0 % flour basis) and water (up to optimum absorption). The ingredients were mixed, proofed and baked according to Sanz-Penella *et al.* [7].

In vitro *starch digestion and GI estimation*

To evaluate the *in vitro* rate of starch hydrolysis was employed the method described by Goñi *et al.* [17] with slight modifications. Briefly, the digestion procedure included a bread sample (100 mg) in HCl-KCl buffer (pH 1.5) with 400 μL pepsin (0.1 g/mL) and constant stirring for 1 hour in a water bath at 40 ºC. The volume was adjusted to 20 mL with Tris-Maleate buffer (pH 6.9). Then, 10 mL of a solution containing α-amylase (equivalent to 48 IU of enzyme activity per gram of sample) in Tris-Maleate buffer (pH 6.9) was added. The samples were incubated at 37°C in a shaking water bath. Aliquots of 1 mL each 0, 20, 40, 60, 90, 120, 180 min were obtained and incubated at 100ºC for 5 min to inactivate the enzyme. Each test was cooled at the end of the incubation time. After centrifugation (10,000 g at 4 ºC) 500 μL of the supernatant were taken to a volume of 2 mL with sodium acetate buffer (pH 4.75). Then, 60 μL amyloglucosidase

(82 mg/mL, equivalent to 330 units) was added and incubated at 60ºC for 45 min with constant stirring. Subsequently, released glucose was determined spectrophotometrically according to a commercially available enzymatic kit (D-Glucose Assay Procedure, K-GLUC 07/11, Megazyme). The rate of starch digestion was expressed as the percentage of total starch hydrolyzed at 0, 20, 40, 60, 90, 120, 180 min. The total starch content was determined by the AOAC official method [18]. Finally, the area under the curve (AUC) from 0 to 120 min and total digestible starch was used to calculate an *in vitro* glycaemic index value normalised against white bread (SigmaPlot software, Version 12.0) expressed as a percentage.

Statistical analysis

Multiple sample comparison of the means (ANOVA) and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Centurion XV.II, and the significance level was established at $P<0.05$.

Results and Discussion

Bran supplementation prevented the proper gluten network formation during proofing [7-9], which decreased the dough height and consequently affecting bread performance in terms of loaf volume and crumb structure (Fig 1). Smaller bran particle size increases the surface contact between bran and gluten network, consequently increase the negative effect on loaf volume [7], whereas α-amylase displayed a positive effect on this parameter (Fig 1). Increasing levels of bran affected negatively the crumb structure and induced a significant decrease in cell/total cell area from 0.19-0.25 to 0.10-0.13 cm^2/cm^2. The number of cells was also reduced from a maximum of 179 to a minimum

of 114 cells/cm^2 and the mean cell area decreased up to 1.5-fold (p<0.05). However, the addition of the enzyme attenuated these differences.

In general, the glycaemic effect of foods depends on the food texture and particle size, type of starch, degree of starch gelatinization, the physical entrapment of starch molecules within food, food processing and other ingredients [19-20]. White bread showed the highest percentage (%) of starch hydrolysis compared to samples with bran (Table 1). Increasing bran proportion rendered lower starch hydrolysis percentages. The extent of hydrolysis in samples with α-amylase supplementation was statistically higher than their respective counterparts without the enzyme. In general, the inclusion of higher amount of wheat bran in formulations provided a significant decrease (p<0.05) in the total hydrolysable starch amount of bread, from 77.4% (white bread) until 68.0-69.7 and 62.0-63.6% (formulations supplemented with 10 and 20% bran, respectively). Starch is one of the major components in wheat bran, and its physicochemical and functional properties relative to wheat starch are different, which could also affect their rate of hydrolysis [21]. In this sense, the latter authors indicated that wheat bran starch contained more resistant starch and amylose, and exhibited a higher crystallinity, swelling power and melting enthalpy, possessed lower gelatinization temperature, pasting peak, final viscosities, and retrogradation rate comparing to wheat starch. Accordingly, bran supplementation in bread formulations produced a significant decrease (p<0.05) in GI, between 12 and 16 units in bread with 10% of bran and, between 19 and 23 units in breads with 20%, compared to the reference (Table 1).

At 90 min white bread showed 71.0% of starch hydrolysis. TSH_{90} (total starch hydrolysed at 90 min) was reduced by 8.0-11.0 and 12.6-15.2% in samples prepared with 10 and 20% bran, respectively, depending of bran particle size distribution (Table 1). It seems that higher particle size favoured a trend to decrease GI values. However,

this difference was not significant (P<0.05) in comparison to GI for samples with lower particle size. Besides, according to the literature, particle size has to be the major factor contributing to the significant differences observed in GIs of similar foods with analogous proximate compositions [22]. Relatively smaller particle size distribution elicited higher GIs compared to food made of stone ground flours, where the particle size was larger [22]. This is supported by findings of previous studies, which suggest that particle size probably exerts its greatest effect on glucose and insulin responses when large food or grain particles are present [23]. On the other hand, Behall *et al.* [24] reported that the particle size of whole grain wheat flour did not substantially affect glycaemic responses. Previous studies have indicated that breads made with different sources of dietary fibre or their mixtures in baked goods exert a hypoglycaemic effect on humans [25-27]. Other studies demonstrated beneficial effects of nondigestible carbohydrates lowering postprandial glucose levels after ingestion of high-glycaemic-index breakfasts in human [28]. This observation has been linked to fibre-mediated decrease of glucose uptake or the hypoglycaemic effect of fermentation-derived products improving insulin sensitivity [28], the production of gut hormones (glucagon-like peptide-1 and peptide YY) or modulating inflammation by their interaction with specific G-protein coupled receptors (GPR43 and/or GPR41) [29]. These beneficial effects of fibres on physiological processes could have important consequences in the health status and disease development as glucose-induced low grade inflammation, together with insulin resistance have been associated to T2D and obesity that can further develop to non-alcoholic fatty liver disease and its more severe form steatosis with inflammation [30].

Inclusion of α-amylase in formulations increased TSH_{90} by 5.5 and 8.2% in samples with low particle size formulated with 10 and 20% bran, respectively, compared to the

reference bread (Table 1). The samples with larger bran particle size displayed an increase in GI up to 10-12 units with the inclusion of amylase compared to their counterparts without the enzyme, but still significantly lower comparing to the reference sample (Table 1). In this study, the addition of α-amylase exerted a positive effect in bread quality but increased the values calculated for GI despite the particle size.

In this study there have been used the widely accepted and established Lineweaver-Burk's plot to calculate the kinetic parameters of the starch hydrolysis (Fig. 2) [31]. The utility of this plot resides in the transformation of cumulative into linear curves from which key information can be obtained (Table 1). This method do not need additional data to those obtained from the cumulative curves, but only to calculate the reciprocal values to [% Starch hydrolysis] and time.

The inclusion of the enzyme was not reflected in higher coefficients of hydrolysis (inverse of *y*-intercept value of Lineweaver-Burk's plot) calculated for the different samples (Table 1 and Fig 2). It is worth to note that α-amylase addition only increased the coefficient of hydrolysis for samples La-10-Am; however, formulation with higher bran proportion counteracted this increase and α-amylase addition did not vary the coefficient of hydrolysis in samples La-20-Am. The particle size appeared as a critical factor abolishing the influence of α-amylase addition in samples La-10-Am.

In all cases there were calculated higher values for the slope of the plotted lines of kinetic Lineweaver-Bürke's transformation of percentages of starch hydrolysis in relation to the WB (white bread as control sample) (Table 1). Thus, sample formulated with 20 % of bran without α-amylase showed the lowest rate of starch hydrolysis, followed by the samples with 10 % of bran without the enzyme (Table 1 and Fig 2). Taken together these results indicate similar starch hydrolysis rates samples added with α-amylase despite the bran proportion used in the formulation, although, the

contribution of bran can reduce the uptake kinetics for glucose with potential significant different physiological effects. Thus, it would be needed to evaluate what could be the physiological consequence because there are significant advantages when fibres are included in bread formulation.

In summary, the use of α-amylase in bread formulations significantly improves the product quality, including texture, shelf life and flavour [8, 11-12]. These advantageous features favour their widespread use in baking industries. The use of α-amylases in bakery products could counteract the functional properties of formulating bakery products with bran and other resistant oligosaccharides. However, the use of flour with a high degree of extraction or high bran amount reduces the GI even with the inclusion of α-amylase in the formulation in relation to WB. Therefore, when formulating breads with bran and α-amylase in order to obtain products with low GI values, it should be taken into account the percentages of both ingredients. The fact that inclusion of α-amylase increases the kinetics of starch hydrolysis urges for safety studies after long-term consumption of these formulations.

Acknowledgements

This work was financially supported by grants Consolider Fun-*C*-Food CSD2007-00063 from the Ministry of Economy and Competitiveness (MINECO) and PROMETEO/2012/064 from the Generalitat Valenciana, Spain. The contract of J.M. Sanz-Penella from MINECO is greatly acknowledged.

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Figure legends

Fig. 1 Effect of bran and α-amylase on bread. Bread formulations: 10 % (10) or 20 % (20) of bran; average diameter 800 μm (Large size, La) or 300 μm (Small size, Sm); fungal α-amylase addition dose 0.5 U Kg^{-1} of flour (Am); volume (V)

Fig. 2 Kinetic Lineweaver-Bürke's transformation of percentages of starch hydrolysis calculated for the different samples analyzed. Slope and Coefficient of hydrolysis calculated for each sample are reported in Table 1. Symbols: ×, White bread; Δ, La-10; ▲, La-10-Am; ◊, Sm-10; ♦, Sm-10-Am; □, La-20; ■, La-20-Am; ○, Sm-20; ●, Sm-20-Am. Bread formulations: 10 % (10) or 20 % (20) of bran; average diameter 800 μm (Large size, La) or 300 μm (Small size, Sm); fungal α-amylase addition dose 0.5 U Kg^{-1} of flour (Am)

Table 1. Effect of wheat bran and α-amylase addition on glycaemic index[a]

Formulation			Total Starch	TSH_{90}	AUC	GI	Coefficient of Hydrolysis[b]	Slope-LB[b]
Bran Particle Size	Bran (%)	Amylase	(%)	(%)			SH/min	min/SH
Control	**0**	**(-)**	77.4 ± 1.6^{a}	71.0 ± 1.8^{e}	11345	100 ± 2^{e}	87 ± 4^{ab}	0.22 ± 0.02^{a}
Large	**10**	**(-)**	69.0 ± 1.1^{b}	60.0 ± 3.1^{ab}	9513	84 ± 2^{bc}	84 ± 6^{ab}	0.43 ± 0.03^{de}
Small	**10**	**(-)**	68.0 ± 0.4^{b}	63.0 ± 2.2^{bc}	10009	88 ± 3^{cd}	86 ± 2^{ab}	0.38 ± 0.02^{cd}
Large	**20**	**(-)**	63.5 ± 0.8^{c}	55.8 ± 1.1^{a}	8699	77 ± 2^{a}	85 ± 12^{ab}	0.60 ± 0.07^{f}
Small	**20**	**(-)**	63.6 ± 0.7^{c}	58.4 ± 1.9^{ab}	9154	81 ± 2^{ab}	79 ± 11^{a}	0.46 ± 0.08^{e}
Large	**10**	**(+)**	68.8 ± 0.3^{b}	67.7 ± 1.0^{de}	10683	94 ± 6de	92 ± 4^{b}	0.31 ± 0.02^{b}
Small	**10**	**(+)**	69.7 ± 0.7^{b}	65.5 ± 1.9^{cd}	10587	93 ± 4^{d}	87 ± 7^{ab}	0.31 ± 0.06^{bc}
Large	**20**	**(+)**	62.5 ± 0.3^{c}	63.0 ± 2.4^{bc}	10129	89 ± 5^{cd}	86 ± 3^{ab}	0.33 ± 0.02^{bc}
Small	**20**	**(+)**	62.0 ± 0.4^{c}	62.8 ± 2.5^{bc}	9980	88 ± 2^{cd}	79 ± 12^{a}	0.35 ± 0.08^{bc}

[a]Total starch hydrolysed at 90 min (TSH_{90}); area under the curve of starch digestion (AUC); glycaemic index (GI). Mean ± Standard Deviation, n=3. Values followed by the same letter in the same column are not significantly different at 95% confidence level. [b]Slope and Coefficient of hydrolysis calculated for each sample using the Lineweaver-Bürke's transformation of the TSH accumulation curves.

Fig 1.

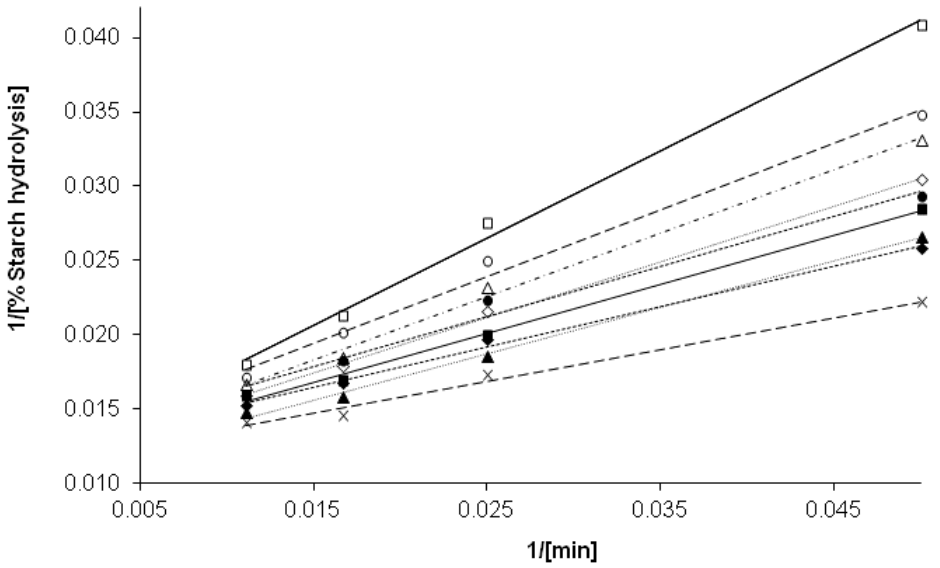

Fig 2.

Conflict of Interest

This article does not contain any studies with human or animal subjects.