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Wheat-based breads with slowly digestible starch properties by increasing the amylose content: an *in vitro* approach

G. Giuberti[a,*], A. Gallo[a], P. Fortunati[a] and F. Rossi[a,b]

[a]*Institute of Food Science and Nutrition, Agriculture, Food and Environmental Sciences Università Cattolica del Sacro Cuore, Piacenza, Italy*

[b]*CriBeNS, Centro di Ricerca in Biochimica e Nutrizione dello Sport, Università Cattolica del Sacro Cuore, Roma, Italy*

Abstract.

BACKGROUND: Previous experiences revealed that increasing the amylose content through the substitution of normal amylose (NA) with high amylose (HA) flours can improve slowly digestible starch properties of cereal-based foods. However, data for wheat-based breads are not clear, since HA sources are frequently used in combination with organic acids and/or specific baking conditions know to reduce rate and extent of starch digestion.

OBJECTIVE: The aim was to conduct an *in vitro* evaluation of starch digestibility of wheat-based breads characterised by increasing amylose levels.

METHODS: Wheat-based breads were formulated with increasing amylose levels derived from the substitution of NA white wheat (NAWW) flour with HA maize starch (HAMS) represented by substitution ratio of 0%, 15% and 30% on a total flour basis.

RESULTS: Dietary fibre increased ($p < 0.05$) whereas crude protein decreased ($p < 0.05$) when the level of HAMS increased in the recipe. The resistant starch and slowly digestible starch fractions increased ($p < 0.05$), whereas the predicted glycaemic index and the *in vitro* rate of starch hydrolysis decreased ($p < 0.05$) when HAMS increased in the formulation.

CONCLUSIONS: Present findings suggested that the use of HA ingredients could contribute to formulate wheat-based breads with overall slowly digestible starch properties.

Keywords: Bread, high amylose, predicted glycaemic index, resistant starch, wheat

1. Introduction

Wheat-based bread represents a traditional staple food, providing energy mainly in the form of starch and several essential nutrients, including dietary fibre, proteins, vitamins and minerals. From a nutritional perspective, it belongs to high glycaemic index (GI) food categories, with great rate of starch digestion and negligible amount

*Corresponding author: Gianluca Giuberti, Institute of Food Science and Nutrition, Agriculture, Food and Environmental Sciences Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29121 Piacenza, Italy. Tel.: +39 0523 599 433; Fax: +39 0523 599 259; E-mail: gianluca.giuberti@unicatt.it.

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of resistant starch (RS) [1]. This may be of concern, since recent health claims support the consumption of foods with slowly digestible starch properties [2].

The GI concept characterizes carbohydrates on the basis of the postprandial level of blood glucose, foods being divided into low (≤55), medium (56–69) or high (≥70) GI categories [3]. Lowering the GI of foods improves glucose and insulin controls in human subjects, having implications in the prevention of obesity and metabolic risk factors [4]. In addition, dietary starch has been classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS according to its enzymatic hydrolysis [5]. The measurement of starch fractions can provide valuable prediction of the rate and extent of starch digestion in human subjects. Accordingly, RDS fraction is rapidly digested and absorbed, leading to a rapid elevation of blood glucose, whereas SDS, going through a slower but complete hydrolysis in the small intestine, does not produce postprandial hyperglycaemic and hyperinsulinemic spikes [6]. Lastly, RS represents the sum of starch and related degradation products not absorbed in the small intestine showing variable fermentation potentials by caecal and colonic microbial communities [7]. The RS is considered a type of dietary fibre and established beneficial effects associated with greater RS intake involve improved metabolic control, prevention of colorectal cancer and marked prebiotic effects [8]. Furthermore, RS possesses a lower calorific value compared with digestible starch (8 versus 15 kJ/g, respectively), thus contributing to reducing the energy content of food products [7].

Main factors responsible for the high GI value and the low RS content of wheat-based bread include: 1) the botanical origin of flours, which determines the structural type of starch and the ratio of amylose and amylopectin; 2) the heat processing, which regulates the extent of starch gelatinisation; 3) the food structure, which influences the susceptibility of starch to enzyme attack [9]. Considering starch composition, it can vary from being virtually amylose-free to being high in amylose, with values greater than 40% on a total starch basis [10]. Several experiences revealed that increase the amylose content through the substitution of normal amylose (NA) with high amylose (HA) flours was directly related to the development of cereal-based foods with slowly digestible starch characteristics [11–13]. However, available data for wheat-based breads are not clear, since HA sources are frequently used in combination with organic acids and/or specific baking conditions know to reduce rate and extent of starch digestion [1]. A better understanding of starch digestion kinetics solely related to this type of substitution is therefore required and, for this purpose, *in vitro* digestion methods can be useful because of a high-correlation with *in vivo* data [14].

The aim of the present study was to conduct an *in vitro* evaluation of the starch digestibility of wheat-based breads characterised by increasing amylose contents derived from different levels of HA maize starch (HAMS) in substitution of NA white wheat (NAWW) flour. Starch fraction contents, along with the classification through predicted glycaemic index (pGI) values were investigated. In addition, potential implications for human health are presented and discussed.

2. Material and methods

2.1. Raw materials, recipes and baking conditions

The HAMS flour (Amylo-maize starch N-400; 65.0% amylose content according to the manufacturer) was obtained from Roquette Italia SpA (Sardigliano Alessandria, Italy). All other ingredients, including dry yeast (PaneAngeli, Italy) and salt with iodine (Salina di Margherita Savoia, Italy) were food grade. Three different wheat-based breads were prepared. For control bread (0-BR) the recipe was based on: 270 g soft NAWW flour (Barilla SpA, Parma, Italy; 25.0% amylose content according to the manufacturer), 2.7 g dry yeast, 2.1 g salt and 180 g water. Part of NAWW flour equivalent to 15% and 30% (on a total flour basis) was substituted with HAMS flour to formulate 15-BR and 30-BR experimental breads, respectively. The substitution levels were selected through preliminary baking trials, 30% HAMS being the maximum level where changes in bread texture characteristics were not noticeable. All ingredients were mixed and baked with a commercial bread

maker machine (OW3101, Moulinex International, Italy) using the basic white bread program (program number 1; medium crust; total time 190 min). After the cycle was complete, products were cooled to room temperature and stored at –18°C in plastic bags. Prior to evaluation, breads were thawed for 24 h at 4 °C and then equilibrated to room temperature for 4 h. Three different batches of 0-BR, 15-BR and 30-BR were produced. Commercial wheat bread (soft white loaf; ingredients: wheat flour, water, olive oil, yeast, salt, sugar, malted barley flour) was used as reference.

2.2. Proximate composition of breads

For each bread, a representative portion was dried at 55 °C for 48 h in a forced-air oven, ground through a 1-mm screen using a laboratory mill (Retsch grinder model ZM1; Brinkman Instruments, Rexdale, ON, Canada) and analysed [15] for dry matter (DM; 930.15), ash (942.05), crude protein (976.05), crude lipid (954.02 without acid hydrolysis), total dietary fibre (Megazyme assay kit K-INTDF 02/15, which includes RS and non-digestible oligosaccharides [16]) and total starch contents (Megazyme assay kit K-TSTA 07/11). For each treatment, batches were analysed in triplicate.

2.3. Determination of starch digestive fractions

The RDS, SDS and RS levels were measured using the Englyst et al. [17] enzymatic protocol. Samples were pre-treated with a 0.05 M HCl solution containing pepsin (5 mg/ml; P-7000, Sigma-Aldrich® Co., Milan, Italy) for 30 min at 37° C under gentle agitation. The pH was then adjusted to 5.2 by adding 20 ml of 0.1 M sodium acetate buffer prior to the addition of an enzyme mixture with an amylase activity of about 7000 U/ml given by pancreatin (about 7500 FIP-U/g; 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/ml; A-7095, Sigma-Aldrich® Co., Milan, Italy) and invertase (about 300 U/g; I-4504, Sigma-Aldrich® Co., Milan, Italy). The glucose released from samples after 20 min and 120 min of incubation was measured colorimetrically using a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy) and converted to RDS and SDS values [5]. The RS was measured as the starch that remained un-hydrolysed after 120 min of incubation [5]. A starch digestible index (SDI), which represents a measure of the rate of starch digestion, was calculated [5]. For each treatment, batches were analysed in triplicate.

2.4. In vitro starch digestion and calculations

The multi-enzymatic method proposed by Granfeldt et al. [18] was employed to evaluate the *in vitro* starch digestion of breads over time. This method has been shown to predict GI values with good accuracy for several cereal and legume foods. For each product, sample portions containing 1 g of available starch were tested. Healthy subjects participated in the chewing phases of the experiment to simulate the oral phase of the digestion [18]. The chewed materials were then carefully transferred into glass tubes containing 0.05 M phosphate buffer adjusted to pH 1.5 with HCl, and the mixture was incubated with pepsin (P-7000, Sigma-Aldrich® Co., Milan, Italy) for 30 min at 37°C with gentle mixing. After neutralisation to pH 6.9 with NaOH, porcine pancreatic α-amylase (A-6255, Sigma-Aldrich® Co., Milan, Italy) was added, the sample was brought to volume with phosphate buffer and then transferred to the dialysis tubing (13 cm strips, Spectra Por No. 2, width 45 mm, molecular weight cut-off 12,000–14,000) and incubated at 37 °C for 180 min in 800 ml of phosphate buffer. Every 30 min, aliquots from the dialysate were removed for analysis of reducing sugars by the 3,5-dinitrossalicylic acid method using a maltose standard curve [19]. The hydrolysis index (HI) was then derived from the ratio between the area under the hydrolysis curve (0–180 min) of breads 0-BR, 15-BR and 30-BR and the corresponding area of the reference sample chewed by the same person. From the HI obtained *in vitro*, the pGI was calculated using the formula $pGI = 8.198 + 0.862 \times HI$ [20].

To describe starch hydrolysis kinetics, a first-order exponential model ($\mathbf{C_t = C_{0+} C_{\infty-0}}$ $(1 - e^{-\mathbf{kt}})$ was applied, where $\mathbf{C_t}$ is the starch hydrolysed at time t (g/100 g dry starch), $\mathbf{C_0}$ is the starch solubilised in the buffer at 0 min (g/100 g dry starch), $\mathbf{C_\infty}$ is the equilibrium percentage of starch hydrolysed at infinite time (g/100 g dry starch), $\mathbf{C_{\infty-0}}$ is $\mathbf{C_\infty}$ minus $\mathbf{C_0}$ (g/100 g dry starch), **k** is the rate constant (min^{-1}) and **t** is the chosen time (min) [21]. For the purpose of data fitting, values were obtained by the Marquardt method using the PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., U.S.A).

2.5. Statistical analysis

Data were analysed as a completely randomised design using the MIXED procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., USA) according to the model:

$$\mathbf{Y_{ij}} = \mu + \alpha_\mathbf{i} + \mathbf{e_{ij}}$$

where $\mathbf{Y}_{ij}$ is the dependent variable on the j th subject (bread batch) assigned to treatment **i**, μ is the overall mean, $\alpha_\mathbf{i}$ is the fixed effect of HAMS substitution to NAWW flour ($i = 0$, 15 and 30%), and $\mathbf{e_{ij}}$ is the residual error. Orthogonal linear contrast was *post-hoc* carried out to study the linear response of fixed effect. Through the text, terms "increased" or "decreased" refer to the linear trend of treatments. Experimental unit was the bread batch. Significance was declared at $p < 0.05$.

3. Results and discussion

3.1. Chemical composition and starch fractions of experimental breads

The chemical composition of experimental breads was influenced by HAMS addition (Table 1). Total starch content increased ($p < 0.05$) from 72.1 g/100 g DM to 73.6 and 74.5 g/100 g DM, whereas crude protein content decreased ($p < 0.05$) from 9.4 g/100 g DM to 7.3 and 6.6 g/100 g DM when the level of HAMS increased in the recipe. A similar pattern has been previously reported for cookies produced by the substitution of HA ingredients for a part of NA wheat flour [13]. The total dietary fibre content increased ($p < 0.05$) from 2.2 g/100 g DM to 3.6 and 5.2 g/100 g DM for control, 15-BR and 30-BR, respectively. Analogously, gluten-free breads prepared with a 20% replacement of NA maize starch with HAMS (on a total starch basis) showed a marked rise by 88% of total dietary fibre content with respect to control samples [22]. Greater amounts of dietary fibre in refined cereal products are favourable, aiming to raise the overall health profile of these types of foods [23].

Concerning starch fractions, both RS and SDS levels increased ($p < 0.05$) from 1.3 g/100 g DM to 4.4 and 6.1 g/100 g DM and from 5.8 g/100 g DM to 21.6 and 27.5 g/100 g DM, whereas the RDS level decreased ($p < 0.05$) from 64.9 g/100 g DM to 47.6 and 40.9 g/100 g DM for control, 15-BR and 30-BR, respectively. The conversion of RDS to SDS and RS fractions can improve the nutritional profile of foods aiming to formulate products with slowly digestion starch properties [24]. Similarly, Agama Acevedo et al. [25] obtained lower RDS and higher SDS contents in bakery products added with HA banana starch when compared to control samples. Present findings corroborate the hypothesis that the formation of RS in bakery products is strongly related to the amylose content [26]. Similarly, higher amounts of RS have been reported for arepa maize bread made from HA genotypes compared with corresponding products prepared with NA maize flours [27]. The hydration of native starch during heat treatment, commonly referred as gelatinisation, markedly increases starch susceptibility to amylolytic degradation. However, higher level of amylose can contribute to obstruct starch hydration, amylose molecules being more compact when compared to amylopectin [28]. This in turn may affect the degree of starch swelling during gelatinisation to such an extent that the overall accessibility of starch to hydrolysing enzymes can be significant lowered [29]. Consequently, the RS fraction in HA breads may correspond to amorphous amylose not gelatinised during normal bread-making conditions, as already indicated by Hoebler et al. [30]. In addition,

Table 1
Chemical composition (g/100 g dry matter), starch fraction contents (g/100 g dry matter) and starch digestible index (%) of wheat-based breads added with different substitution levels of high amylose maize starch (HAMS) flour

Parameters	Substitutions with HAMS flour			$\sqrt{MSE}$	*p*-value
	Bread 0-BR	Bread 15-BR	Bread 30-BR		
Chemical composition					
Total starch	72.1	73.6	74.5	0.97	<0.05
Crude protein	9.4	7.3	6.6	0.35	<0.05
Crude lipid	2.0	1.9	1.7	0.07	n.s.
Dietary fibre	2.2	3.6	5.2	0.51	<0.05
Ash	1.5	1.4	1.2	0.08	n.s.
Starch fractions					
Resistant starch	1.3	4.4	6.1	1.17	<0.05
Rapidly digestible starch	64.9	47.6	40.9	2.56	<0.05
Slowly digestible starch	5.8	21.6	27.5	2.55	<0.05
Starch digestible index	90.1	63.3	52.2	2.49	<0.05

Abbreviations: 0-BR, control bread formulated with 0 : 100 HAMS:normal amylose white wheat (NAWW) flour; 15-BR, bread formulated with 15 : 85 HAMS:NAWW; 30-BR, bread formulated with 30 : 70 HAMS:NAWW; n.s., not significant ($p > 0.05$).

on cooling after gelatinisation, part of the starch may undergo a re-association process called retrogradation. This process can produce firmly recrystallized structures which contribute to form RS, higher amounts of amylose being usually linked to a greater and much faster retrogradation tendency than amylopectin [31]. However, at the highest inclusion level of HAMS, the RS content of 30-BR was about 8% of the total starch content, lower than the minimum RS value (i.e., 14 %) proposed for baked goods [2]. Lastly, the SDI decreased ($p < 0.05$) from 90.1 to 63.3 and 52.2 with increasing HAMS levels in the recipe. Accordingly, Englyst et al. [5] reported a SDI value for white wheat bread of 90, whereas a SDI of 56 was obtained for maize RS products.

3.2. Predicted glycaemic index and in vitro starch digestion kinetics of experimental breads

The classification through a pGI is useful to predict the likely *in vivo* glycaemic response of a food of interest, this parameter being positively related to *in vivo* GI values for a wide number of food products [18]. As reported in Table 2, both HI and pGI decreased ($p < 0.05$) from 102 to 66 and from 96 to 65 when the HAMS increased in the composite. The pGI of the reference sample was similar to that measured for 0-BR (94 vs. 96, respectively). The lower pGI recorded for HAMS-enriched breads could be related to their respective SDS and RS contents, both fractions associated with decreased *in vivo* and *in vitro* glycaemic responses [27, 32]. Similarly, Hallström et al. [1] showed that wheat breads containing high amounts of RS (4.4 g/100 g DM) elicited the lowest glycaemic response when compared to other test products. In addition, present findings seem to confirm previous indications that the RS fraction may influence not only the amount of unavailable starch but also the digestibility of available starch itself, due to a possible encapsulation of gelatinised starch between RS layers [31].

Lastly, slower *k* values ($p < 0.05$) were calculated when the HAMS level increased in bread formulation, with values ranging from 0.176 min^{-1} to 0.075 min^{-1} and 0.041 min^{-1} for 0-BR, 15-BR and 30-BR, respectively. Previous experiences revealed that the rate of *in vitro* starch hydrolysis corresponded well with the *in vivo* rate of starch uptake, the latter considered as one of the major determinants of the glycaemic response, faster starch digestion rates being usually associated with greater extent of starch digestion [9, 32]. The reduction in k values may be related to the variation in the proportion of amorphous starch material due to the presence of different HA levels in the composite. In addition, the possible formation of complexes between amylose and surrounding

Table 2
Starch hydrolysis index (HI), *in vitro* predicted glycaemic index (pGI) and fitted kinetic parameters (C_0 and C_∞, g/100 g dry starch and k, min^{-1}) of wheat-based breads added with different substitution levels of high amylose maize starch flour (HAMS)

Parameters	Substitutions with HAMS flour			$\sqrt{MSE}$	p-value
	Bread 0-BR	Bread 15-BR	Bread 30-BR		
HI*	102	88	66	1.65	<0.05
pGI**	96	84	65	1.41	<0.05
Fitted kinetic parameters					
C_0	2.44	2.32	2.06	0.78	n.s.
C_∞	96.6	87.8	69.3	3.09	<0.05
k	0.176	0.075	0.041	0.0162	<0.05

Abbreviations: 0-BR, control bread formulated with 0 : 100 HAMS:normal amylose white wheat (NAWW) flour; 15-BR, bread formulated with 15 : 85 HAMS:NAWW; 30-BR, bread formulated with 30 : 70 HAMS:NAWW; $\mathbf{C_0}$, starch solubilised in the buffer at 0 min; $\mathbf{C_\infty}$, potential digestibility of starch; k, *in vitro* rate of starch digestion. n.s., not significant ($p > 0.05$). *Calculated using commercial white wheat bread as reference (HI = 100). **Calculated with the equation of Granfeldt [20] (pGI of the reference bread = 94).

lipids and proteins during baking may be additional factors contributing to lower the rate of starch hydrolysis in products with elevated amylose content [31].

Taking together, present *in vitro* findings indicated that increase the amylose content through the substitution of NA with HA flours can enhance the overall slowly digestible starch properties of wheat-based breads, in agreement with current dietary indications [2]. Beyond the different amylose content of selected ingredients, structural characteristics of the starches used should be considered. Firstly, with respect to wheat starch, maize starch is characterised by larger size of starch granules [33]. A smaller surface area to volume ratio differentiates larger starch granules and thus a lower surface potentially subjected to enzyme hydrolysis [9]. Secondly, starch granules display different types of crystalline domains that resist enzyme hydrolysis to different degrees [14]. In particular, starch from cereal grains exhibits the A-type power diffraction pattern, which has an open structure that renders starches highly digestible, whereas tubers and HA starches give the so-called B-pattern, which is more resistant to digestion due to a close-packed arrangement [9].

3.3. Potential implications for human health

Current *in vitro* findings indicated that the use of flours with starch characterised by higher amylose content could favourably be used in wheat-based bread preparation aiming to increase slowly digestible starch properties. Foregoing results can therefore provide us the opportunity to discuss additional aspects in relation to human nutrition and health. In general, carbohydrate foods vary in their potential to provoke different postprandial glycaemic response in humans. This response has been quantified in several ways, by using both *in vivo* and *in vitro* approaches, including the GI assessment along with the quantification of nutritionally important starch fractions [5, 9]. Although there is an on-going debate, there is general consensus that the reduction of the dietary glycaemic response following the consumption of lower GI and higher SDS and RS content foods can favourably influence physiological parameters implicated as markers for conditions including overweight and obesity, diabetes mellitus and risk of coronary heart disease [2, 4, 6, 8, 34]. For instance, in the long term the consumption of foods with low GI and high unavailable carbohydrate (UC) content improved blood glucose control in patients with type 1 diabetes [34, 35]. Similarly, there is evidence that lower GI and higher UC in diets can reduce the levels of glycated proteins for individuals with fasting blood glucose concentrations in excess of 5 mmol/l [34]. On the other hand, health benefits associated with SDS consumption are mainly related to stable glucose metabolism, diabetes management and improved satiety [6, 36]. Golay et al. [37] demonstrated that

switching, at breakfast only, from standard cereals to foods containing SDS improved carbohydrate metabolism and reduced insulin requirement of insulin-treated type 2 diabetic individuals. A delayed return of hunger state has also been reported in human subjects after the consumption of SDS compared to RDS food sources [38]. Last, the replacement of digestible starch with RS has been proven to decrease post-prandial glycaemic and insulinaemic responses more than the addition of RS itself [2]. This starch fraction provides the colonic microbiota with a fermentable carbohydrate substrate, promoting higher proportion of butyric acid than other indigestible carbohydrates. Butyrate constitutes the major energy substrate for colonocytes and is usually associated with several benefits in relation to large intestine health [7, 8]. To promote aforementioned beneficial effects, the suggested RS intake level was set at about 20 g/person/day [39]. Considering that the average bread ingestion is about 150 g/person/day for European Countries [40], the consumption of experimental breads formulated with the highest HAMS inclusion (i.e., BR-30) could account for more than 50% of the suggested dietary RS intake.

4. Conclusions

Differences in the chemical composition and in the overall *in vitro* starch digestibility were observed in wheat-based breads produced under classical baking conditions and prepared with increasing levels of HA maize starch. Lower protein and higher total dietary fibre and starch contents were achieved when the level of HA maize starch increased in the recipe. From a nutritional standpoint, higher RS and SDS, along with lower RDS and pGI values were obtained when amylose increased in the composite through HA maize starch addition. In order to obtain a deeper characterisation of current-developed food products and to verify possible human health benefits related to their consumption, a complete sensory evaluation along with an *in vivo* assessment are strongly warranted.

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