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Enzymatic Wheat Conditioning

Alice Gruppi, Guillermo Duserm Garrido, Roberta Dordoni, Dante M. De Faveri, Giorgia Spigno*

Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy
giorgia.spigno@unicatt.it

This work was aimed to the preliminary development of an enzymatic wheat conditioning process in order to partly hydrolyse the fibre fraction to increase both milling yield and antioxidant capacity of whole flour. Two different commercially available food grade enzyme preparations were selected and used: Viscozyme® L (a complex including several carbohydrase activities) and a 50:50 mixture of Celluclast® BG and Fungamyl® Super AX (a mixture of purified 1,4-β-xylanase and a fungal α-amylase). Lab-scale conditioning trials were carried at 16 % final grain moisture, under stirred conditions for 24 h. The investigated process variables were: working temperature (25 °C and 40 °C) and enzyme addition (3 % w/w based on dry weight of wheat, at 25 °C). All the samples and a control (not conditioned grain), were ground in a domestic mill and the flours were analysed to evaluate the influence of the treatment on: friability of bran fraction (flour weight fraction with particle size < 0.5 mm); free glucose and xylose content; total dietary fibre content; total free phenols (expressed as equivalents of ferulic acid based on Folin's assay) and antioxidant capacity (according to different *in vitro* test: ABTS, FRAP and ORAC assay). Use of enzymes significantly increased the bran friability since the % of flours with particle size < 0.5 mm was 14 % higher than in the samples conditioned only with water. The free glucose content was 350 % higher in the enzymatically treated flours, while free xylose was detected only in the flour treated with the Celluclast®-Fungamyl® mixture. The total dietary fibre content was decreased by the Viscozyme® treatment. Both the enzymatic treatments improved the antioxidant capacity of flour based on ORAC and FRAP assays, but not the total phenols content and the ABTS-antioxidant capacity. The results showed an effect of the enzymes on the cell wall components, suggesting interesting potential for the development of an enzymatic wheat conditioning process.

1. Introduction

Over the last years, the market demand for high fibre foods has been constantly increasing following the overall new challenge for the society to promote a long, healthy, and active life span (Minuti et al., 2014). "Source of fibre" and "High fibre" are permitted EU nutrition claims (EU Reg. No 1047/2012) for products containing, respectively, at least 3 g or 6 g of fibre per 100 g or 1,5 g or 3 g of fibre per 100 kcal.
That is why the industry has focused on the development of high fibre products of typical large daily consumption, such as cereal based products where, on the other hand, the use of a whole flour with a high fibre content may typically impair technological (such as machinability and leavening strength) and sensorial problems (such as off-flavors) (Delcour et al., 2012). Another potential negative aspect of whole flour use is the average higher content of unhealthy compounds, such as heavy metals, toxins and residues of pesticides (Moncalvo et al., 2016). Cereal milling processes have in fact been developed to remove the external layers (the bran) of the grains to improve both the technological quality and safety profile of flours. On the other hand, besides the already cited fibre nutrition claim, the external layers contain other healthy compounds, such as lignans, tocotrienols, minerals and phenols (Oghbaei and Prakash, 2016).
In wheat milling, conditioning is traditionally applied before grinding to enhance the separation of the germ and of the external layers from the starchy and proteinaceous endosperm which constitute the refined flour. Conditioning (also referred to as tempering) is the process of adding small amount of water to wheat (to increase the water content to an average 15.5-16.5 %) to toughen the bran and mellow the endosperm and thus improve the efficiency of flour extraction. This step is fundamental also for the technological quality of the

flour, since a complex system of chemical-physical-enzymatic changes occur, partly not yet completely known (Kweon et al., 2009). It is a very critical step to be controlled, because many parameters have to be optimized depending on the wheat type such amount of water to add, soaking time and temperature. Process times can be longer than 24 h, involving large volume vessels and representing a time-cost-consuming operation for the milling industry.

The use of enzymes hydrolysing the bran cell wall components has been investigated in literature for different purposes. Coda et al. (2015) reviewed enzymatic processing of bran and whole grain or bran-enriched flours aimed at enhancing the nutritional and technological functionality of bran. Enzymatic action can increase the amount of soluble dietary fibre but also of hydroxycinnamic acids, free phenols concentration, water soluble antioxidant activity and phenols compounds availability. The largest part of the phenols in cereals are, in fact, present in a form bound to the cell walls (Liyana-Pathirana et al., 2006).

Enzymes addition is a current practice as flours improvers (Ulvskov et al., 2011) playing a role in dough development. They are then added to flours after milling process. Also the recently innovative whole flour launched on the Italian market, FarinaIntera® with an improved nutritional profile, is obtained through a patented enzymatic treatment of the bran separate with milling. Enzymes addition to the tempering water has been less investigated and with the aim of improving milling and baking performance (Haros et al., 2002; Yoo et al., 2009). International enzymes producing companies have started developing specific enzyme mixtures for milling (as Powermill™ by Danisco-DuPont) but enzymatic milling is still far from a consolidate industrial implementation.

Based on these premises, this work was aimed at investigating the development of an enzymatic wheat conditioning process with the potential achievement of different objectives: reduction of conditioning time and/or costs; reduction of energy consumption in the subsequent milling steps; increase in milling yield; modification of the fibre fraction composition to obtain a whole wheat flour with increased amount of soluble dietary fibre, free phenols and antioxidant capacity.

2. Materials and Methods

The soft wheat used in the study was kindly provided by OCRIM SpA (Cremona, Italy).

Two different commercially available food grade enzyme preparations (by Novozyme Corp.) were selected on the base of their activity and literature demonstrated efficiency on wheat bran. The first preparation (E1) consisted of Viscozyme® L (Moore et al., 2006) and that includes several carbohydrase activities, such as arabinase, cellulase, β-glucanase, hemicellulase and xylanase, with declared activity ≥ 100 BGU/g (units of β-glucanase). The second preparation (E2) consisted of a 50:50 mixture of Celluclast® BG (≥ 700 BGU/g) and Fungamyl® Super AX (Messia et al., 2016). Celluclast® BG acts on cellulose, Fungamyl® Super AX is a mixture of purified 1,4-β-xylanase and a fungal α-amylase.

2.1 Tempering

Wheat samples (50 g) were tempered by adding the adequate amount of water (based on wheat moisture content) to 16 % moisture in closed 250 mL flasks and keeping in thermostatic rotary incubator (Infors HT) for 24 hours at 150 rpm. The following tempering conditions were investigated: water at 25 °C; water at 40 °C (in this case it was preliminary verified the amount of water required to compensate water evaporation during incubation); water with E1 at 25 °C; water with E2 at 25 °C. Enzyme preparations were dissolved in the tempering water to have a 3 % w/w based on wheat dry weight. Not tempered grain was used as control.

All the samples were milled in a domestic electric mill (Fidibus Medium Komo) and characterised for: moisture content (by oven drying at 105 °C for 24); weight % of flour with particle size < 0.5 mm (by sieving on a 0.5 mm sieve); free xylose ad glucose content; free total phenols content; total dietary fibre content and antioxidant capacity.

2.2 Analytical methods

- ✓ Free D-xylose and D-glucose content: flour was extracted with water (Karkacier et al., 2003) and the extract was analysed with enzymatic kits (Megazyme, K-FRUGL 09/13 and K-XYLOSE 11/12).
- ✓ Total dietary fiber content of flour was measured with the Megazyme TDF enzymatic kit.
- ✓ Free total phenols content of flour was evaluated by Folin-Ciocalteau analysis, expressed as ferulic acid equivalents (FAE) based on calibration curve with standard ferulic acid (Vellingiri et al., 2014). Preliminary extraction trials were carried out to select the best extraction solvent among aqueous 60 % ethanol (Spigno et al., 2015), 80 % ethanol (Zörb et al., 2007), 80 % methanol (Kang et al., 2016) and acidified 80 % methanol (Ma et al., 2016). The flour (5 g) was extracted with 30 mL of specific solvent in the rotary incubator at 40 °C, 250 rpm for 1.5 h. The surnatant was separated by centrifugation (9500 g) and analysed for FAE content. Based on the obtained results (Fig. 1), 60 % v/v ethanol was selected.

- ✓ Antioxidant activity was evaluated according to three different tests. In the radical ABTS test (García et al., 2011) the results were expressed by means of calibration curve with Trolox® as $\mu mol_{Trolox}/g_{dw}$ (based on wheat dry weight) and as mol_{Trolox}/mol_{FAE} (based on total phenols content). In the FRAP test (Vellingiri et al., 2014) the results were expressed as $\mu mol_{Fe(II)}/g_{dw}$ and as $mol_{Fe(II)}/mol_{FAE}$. In the ORAC assay (Huang et al., 2002) the results were expressed as $\mu mol_{Trolox}/g_{dw}$ and as mol_{Trolox}/mol_{FAE}.

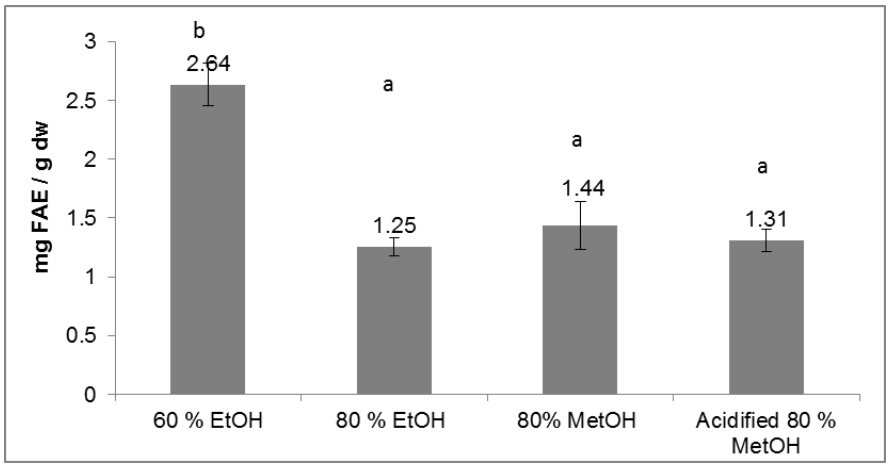

Figure 1: Total phenols extraction from unconditioned wheat flour with different solvents. Error bars indicate ± SD of mean values. Same letter indicates means not statistically different according to ANOVA and Tukey's post-hoc test (p<0.01). FAE: ferulic acid equivalents; dw: dry weight.

2.3 Statistics

All the tempering trials and the analytical measurements were carried out in triplicates (except for total dietary fibre content and % of flour < 0.5 mm, which were evaluated in duplicate). The values are reported as means ± SD. The significance of the influence of conditioning treatment on the measured parameters, was assessed by one-way ANOVA (IBM SPSS Statistics v.23) and Tukey's post-hoc test for means discrimination at a confidence level of 99 % ($p < 0.01$).

3. Results and Discussion

Evaluation of the weight percentage of milled flour with a particle size < 0.5 mm was used as a rough, laboratory estimation of the bran layer friability. Tempering is aimed to toughen the bran so that it can be more easily grinded in small particles. Tempering and addition of specific enzymes for the hydrolysis of fibre component is expected to enhance the friability of outer layers with a reduction in the fraction of coarse particles in the flour. The results reported in Fig. 2 confirmed that tempering, independently of the working temperature, increased the percentage of fine particles and showed that both the enzymatic preparations allowed for a further 13 % increase of the fine fraction. This could lead in the mill to higher milling yield and/or reduced energy consumption for the grinding step.

The total dietary fibre content of final flour was not statistically influenced by the conditioning treatment, except for E1 which gave almost a half content and slightly for E2 (Table 1). The enzymes might have released simple sugars from cellulose and hemicellulose reducing the total dietary fibre value.

Analysis of the content of free monosaccharides confirmed this hypothesis (Table 1). The free glucose content greatly increased with enzymatic tempering. Assuming that glucose is released only from cellulose and considering the 0.88 conversion coefficient from glucose to cellulose (Spigno et al., 2008), it can be calculated that E1 and E2 led to a 1.1 % hydrolysis of wheat dry weight.

Free xylose, on the opposite, was detected only in the flour from E2 tempering suggesting that hemicellulose could be hydrolysed only with this enzymatic preparation. This might be correlated to the Fungamyl® component of E2 containing a highly purified β-xylanase. Also in this case, assuming that xylose is released from hemicellulose and considering the 0.90 conversion coefficient from xylose to xylans (Spigno et al., 2008), it can be calculated that E2 led to a 1.3 % hydrolysis of wheat dry weight.

The calculated reductions cannot explain the reduction in total dietary fibre for the E1 treatment. Evaluation of the release of digestible oligosaccharides may help in future work to better understand the enzymatic action.

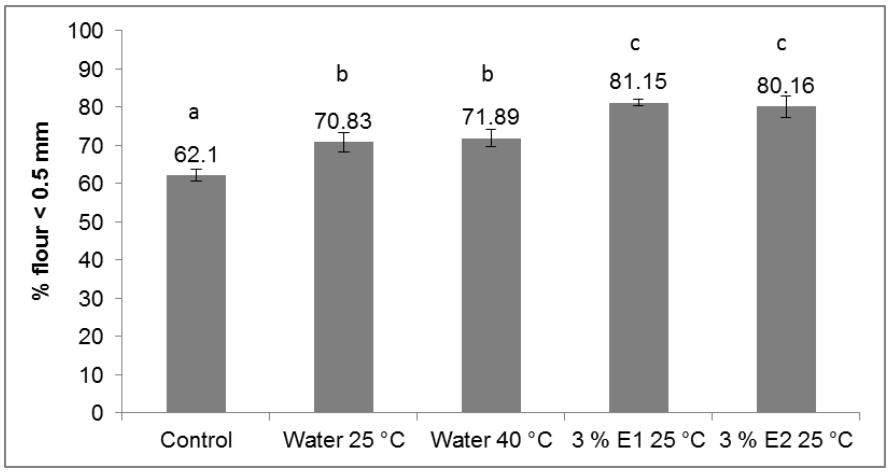

Figure 2: Weigh percentage of flour with particle size < 0.5 mm for flour samples obtained from control grain (not conditioned), and grains conditioned for 24 h with only water at 25 ° or 40 °C, or at 25 °C with 3 % w/w of different enzymatic preparations (E1: Viscozyme®; E2: 50:50 Celluclast® BG and Fungamyl® Super AX) dissolved in water. Error bars indicate ± SD of mean values. Same letter indicates means not statistically different according to ANOVA and Tukey's post-hoc test (p<0.01).

Table 1: Total dietary fibre content of flours obtained from wheat after different conditioning treatments at 24 h with only water at 25 °C or 40 °C, or at 25 °C with 3 % w/w of different enzymatic preparations (E1: Viscozyme®; E2: 50:50 Celluclast® BG and Fungamyl® Super AX) dissolved in water. Same letter indicates means not statistically different according to ANOVA and Tukey's post-hoc test (p<0.01) for the same parameter. dw: dry weight; FAE: ferulic acid equivalents; nd: not detected with detection limit 0.002 mg/g.

Conditioning	Total Dietary Fiber % w/w			Glucose mg/g_{dw}			Xylose mg/g_{dw}		Total Phenols mg_{FAE}/g_{dw}		
	mean	± SD		mean	± SD		mean	± SD	mean	± SD	
None	14.45	0.60	*b*	3.15	0.11	*a*	nd		2.64	0.17	*b*
Water, 25 °C	11.75	0.19	*ab*	4.51	0.12	*c*	nd		3.89	0.14	*d*
Water, 40 °C	11.88	0.46	*ab*	3.78	0.01	*b*	nd		2.09	0.17	*a*
E1 , 25 °C	6.20	1.65	*a*	17.47	0.16	*d*	nd		3.63	0.22	*d*
E2, 25 °C	10.24	1.64	*ab*	17.98	0.18	*e*	13.93	0.14	3.07	0.14	*c*

The free total phenols content was unexpectedly not influenced by enzymes (Table 1). The results seem to indicate that tempering at 25 °C increased the phenols release compared to control grain, while tempering at 40 °C led to a reduced phenols content. Since the treatment lasted for 24 h, released phenols might have been slightly oxidised at this temperature (the Folin assay used for phenols quantification actually measures the reducing power of a sample). Based on this result and on economic considerations for industrial implementation, enzymatic tempering was tested only at 25 °C. Addition of enzymes to the watering temperature, in spite of the previously demonstrated action on cellulose and hemicellulose, did not enhance phenols release. It might be that the enzymes were not able to hydrolyse the ester and ether linkages that the phenolic acids are reported to form with cell wall macromolecules owing to their bifunctional nature through reactions involving their carboxylic and hydroxyl groups (Liyana-Pathirana et al., 2006). Further research to analyse the phenolic profile of the flour extracts by HPLC could help to clarify if the free phenols were not influenced by enzymes or if the Folin's assay could not account for that.

The free phenols extracts of the different flour samples were analysed for the antioxidant capacity with different *in vitro* assays (Table 2).

The ABTS assay is based on antioxidant mechanisms which involve electron transfers (ET), even though adequately carried out can be used to clarify whether the antioxidant reactions are dominate by ET rather hydrogen atom transfer (HAT) mechanisms (Schaich et al., 2015). According to the ABTS assay and in agreement with the total phenols results, the tempering conditions did non influence the antioxidant capacity of the extractable flour components ($\mu mol_{Trolox}/g_{dw}$). However, expression of the ABTS capacity as specific phenolic capacity (mol_{Trolox}/mol_{FAE}) did not gave constant values, showing a poor correlation between

antioxidant capacity and phenols content. As commented above, analysis of phenolic profile could help to understand if different phenols are extracted depending on the tempering treatment.
Even though also the FRAP assay is based on ET reaction, this test could better discriminate the samples, showing a positive effect of both the enzymatic treatments in increasing the amount of free phenolic compounds, based on the values of $\mu mol_{Fe(II)}/g_{dw}$. A poor correlation between FRAP activity and total phenols content was again observed considering the $mol_{Fe(II)}/mol_{FAE}$ ratio which was not constant for the different samples.
Finally, the ORAC assay, although being an HAT based test , showed similar results to FRAP.

Table 2: Antioxidant capacity of flours obtained after different wheat conditioning treatments for 24 h: with only water at 25 °C or 40 °C; or at 25 °C with 3 % w/w of different enzymatic preparations (E1: Viscozyme®; E2: 50:50 Celluclast® BG and Fungamyl® Super AX) dissolved in water. Same letter indicates means not statistically different according to ANOVA and Tukey's post-hoc test (p<0.01) for the same parameter. dw: dry weight; FAE: ferulic acid equivalents.

Conditioning	ABTS assay					
	$\mu mol_{Trolox}/g_{dw}$			mol_{Trolox}/mol_{FAE}		
	mean	± SD		mean	± SD	
None	6.89	0.32	*b,c*	0.51	0.02	*c*
Water, 25 °C	5.41	0.22	*a*	0.27	0.01	*a*
Water, 40 °C	7.62	0.20	*c*	0.70	0.02	*d*
E1 , 25 °C	6.62	0.40	*b*	0.35	0.02	*b*
E2, 25 °C	7.34	0.43	*b,c*	0.46	0.02	*c*
Conditioning	FRAP assay					
	$\mu mol_{Fe(II)}/g_{dw}$			$mol_{Fe(II)}/mol_{FAE}$		
	mean	± SD		mean	± SD	
None	6.92	0.20	*b*	0.45	0.03	*b*
Water, 25 °C	5.34	0.18	*a*	0.27	0.01	*a*
Water, 40 °C	7.48	0.29	*b*	0.69	0.02	*d*
E1 , 25 °C	12.56	0.51	*d*	0.67	0.04	*d*
E2, 25 °C	9.24	0.45	*c*	0.58	0.03	*c*
Conditioning	ORAC assay					
	$\mu mol_{Trolox}/g_{dw}$			mol_{Trolox}/mol_{FAE}		
	mean	± SD		mean	± SD	
None	29.95	4.06	*b*	2.27	0.19	*b*
Water, 25 °C	23.10	3.78	*a*	1.15	0.19	*a*
Water, 40 °C	22.31	2.69	*a*	2.88	0.35	*d*
E1 , 25 °C	41.80	6.73	*c*	2.26	0.34	*b,c*
E2, 25 °C	39.80	4.72	*c*	2.52	0.30	*c*

4. Conclusions

The present study wanted to investigate the application of cell wall hydrolysing commercial enzymatic preparations in a conventional soft wheat tempering process. For this reason, typical industrial tempering conditions, low water content, room temperature and a 24 h duration, were selected for this preliminary experimentation. The used enzyme products contained different carbohydrase activities (such as xylanase and β-glucanase) and the results showed their effect on cellulose and hemicellulose components, since free glucose and xylose content increased in flours obtained from enzymatically treated grain. Reduction in total dietary content was significantly reduced only for one of the enzymatic conditioning. In cereals phenols are mainly present in a form bound to cell wall macromolecules, then the enzymatic treatment was expected to increase the content of free phenols in the flours. Folin's analysis for total phenols quantification did not show any significant increase in free phenols, but the FRAP and ORAC assays for antioxidant capacity evaluation indicated an increase in the content of free bioactive compounds in flours.
The enzyme action on wheat bran led to an increased friability of bran since flours from enzymatically treated grains revealed an increase in the content of smaller particles. This could help in reducing energy consumption in industrial milling allowing also better preservation of flour nutritional profile.
Further investigation is required to get better insight into the enzyme actions and to develop optimised processes for real industrial implementation.

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