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Modification of the molecular properties of wheat grain storage proteins by ozonation

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Abstract

In this study, we report the effects of ozone treatment of different bread wheat grains on the molecular distribution of the storage proteins and their effects on alveographic properties of the flours from ozonated grains. Two wheat cultivars differing on their HMW-GS composition and technological properties were used. Protein solubility evaluated by the Extractable Protein/Total Protein ratio significantly decreased with the ozone treatment and remarkable protein polymers/aggregates were observed in both reduced and non-reduced SDS-PAGE patterns. In addition, ozonated samples displayed increased molecular weight distribution as determined by an A-FFFF method. Protein oxidation by ozone (via disulfide-sulfhydryl interchange reactions and formation of tyrosine bonds) is the main suggested phenomenon which could explain these modifications. All these protein structure modifications have an important effect on bread-making quality of wheat flour (i.e. increase in tenacity and decrease in extensibility of dough).

Key-words: Wheat grain, ozone treatment, storage proteins.

Introduction

Ozone is a powerful oxidizing agent highly reactive which finds increasing applications in many different fields. In 1997, ozone was recognized by U.S. Food and Drug Administration (FDA) as a GRAS (i.e. Generally Recognized as Safe) substance for use as a disinfectant or sanitizer in food industry^[1]. Since this date, the number of O₃ applications did not stop increasing in the field of the production of the major agricultural raw materials (grains, fruits, herbs, spices...)^[2]. As mentioned in these various works, the ozonation of these different agricultural raw materials answers above all to a general concern of food safety (i.e. reduction in the microorganism and virus charge, elimination of various contaminants (residues of pesticides, mycotoxins...)).

However, due to its strong oxidizing properties, ozone is not universally beneficial because, in some cases, O₃ can promote oxidation and/or degradation of chemical constituents present in the treated material (i.e. proteins, lipids, starch). For grains of soft wheat, these molecular alterations can be at the origin of modifications of the technological properties (i.e. rheological properties of product in particular)^[3,4]. However, few published works have investigated the origin of these modifications [i.e. nature of interaction of ozone with the key biopolymers (storage proteins and starch polysaccharides)] present in these biological environments. The objective of this study is to investigate the major effects of ozone treatment on the molecular properties of wheat grain proteins.

Material and methods

Plant material and experimental design

Two bread wheat cultivars were used in this study (Rubisko and Diamento) differing on their HMW-GS composition (7-8+2-12 and 7-9+5-10 respectively). All ozone treatments were conducted with a specific 15 kg semi-batch reactor. A 3-factor 3-level factorial Box-Behnken experimental design (table I) was employed to investigate the effects of selected variables: (X_1) humidification rate, (X_2) inlet ozone concentration and, (X_3) reaction time.

Alveographic measurements

Alveographic measurements were realized following the NF ISO 5530-4 standard.

Quantification of un-extractable polymeric proteins (UPP)

Flour samples (120 mg) were dispersed and incubated at 60°C for 2 hours with 12 mL of 0.05M sodium phosphate buffer (pH 6.9) containing 2% (w/v) SDS, with constant agitation. After centrifugation during 30 min at 12,500×g at 20°C, the supernatant, containing the extractable fraction (EP) was removed. The pellets, containing the un-extractable polymeric proteins (UPP), were kept at -20°C for 24hr, freeze-dried for 48h and their protein contents were determined by the DUMAS method. The extractable Protein (EP) fraction can be determined by using the formula $EP = TPC - UPP$.

Molecular Weight Distribution (MWD) of polymeric proteins

Sample preparation has been conducted according to Lemelin and *al.*^[5]. An AFFFF/MALLS method was used to characterize the polymeric proteins [Eclipse3 F System serially connected to a UV detector (214 nm), a MALLS detector and, an interferometric refractometer]. Sodium phosphate buffer (0.05 M, pH 6.9) containing 0.1% (w/v) SDS was used as mobile phase.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The protein extracts were electrophoresed according to Laemmli^[6] in vertical SDS-polyacrylamide gel electrophoresis slabs at a gel concentration of 10% in a discontinuous, pH 6.8-8.8, Tris-Glycine-SDS buffer system.

Glutenin free sulphhydryl (SH) group determination

Procedure used was performed according to Rakita and *al.*^[7]. Results were calculated against a L-glutathione reduced standard curve.

Ferulic acid extraction and quantification

Extraction of free and bound phenolic acids was conducted according to Zhang and *al.*^[8]. Extracts were then analyzed using a HPLC system composed of a Surveyor LC System coupled to a Surveyor PDA (Thermo Fisher Scientific). A Phenomenex analytical column (250 mm × 4.60 mm, 5 μm, 100 Å) was used. Solvents for the separation were: solvent A, deionized water with 1% (v/v) acetic acid ; and solvent B, acetonitrile (ACN, HPLC grade). Solvents flow rate was maintained at 1.0 mL/min. The phenolic acids were eluted with a 70 min multistep gradient program: 15% B for 40 min, from 15 to 50% B for 5 min, 50% B for 3 min, from 50 to 100% B for 5 min, 100% B for 5 min and, from 100 to 15% B for 5 min. The column was equilibrated at 15% solvent B for 7 min between runs. The column effluent was monitored at 280 nm and 320 nm using a UV detector. The injection volume was fixed at 25 μL. Identification and quantification of ferulic acid in samples were performed by comparison with chromatographic retention times and areas of ferulic acid standard, purchased from Sigma-Aldrich (Sigma-Aldrich Corporation).

Results and discussion

SDS Protein solubility and molecular weight distribution (MWD) of polymeric proteins

As it can be seen in table I, the ozone treatment has significantly modified the 2% SDS solubility of flour proteins which can be evaluated by the UPP/EP ratio. For example, for the cultivar Rubisko, this ratio increases from 0.34 to 0.58 for the control and the run17 (+1/+1/+1) respectively. The same observation can be done with the cultivar Diamento (increase of UPP/EP ratio from 0.63 to 0.97 for the control and the run17 respectively) (data not shown). Even if, experimental conditions (i.e. plant material, variable amplitude...) used in previous works^[9-11] are very different, the results obtained are in total agreement with our observations.

In the same time, the ozone treatment has significantly modified the MWD of flour proteins which can be mainly evaluated by M_n and M_w and this, whatever the wheat cultivar (i.e. HMW-GS composition) studied. Both the number-average molar mass (M_n) and the weight-average molar mass (M_w) of the polymeric proteins very significantly increased [from 9.38×10^5 g.mol⁻¹ and

$3.81 \times 10^7 \text{ g.mol}^{-1}$ to $15.34 \times 10^5 \text{ g.mol}^{-1}$ and $6.77 \times 10^7 \text{ g.mol}^{-1}$ for M_n and M_w of the control and the run17 (+1/+1/+1) respectively] as the result of the grain ozonation. These modifications induced by the ozone treatment can be generalized to all wheat cultivars even if we have to take into account the initial MWD differences (i.e. M_n and M_w of controls) due to the allelic variations coded in particular by the *Glu-1D* locus^[12]. Actually, the presence of the 5 and 10 HMW-GS from Diamento increases the initial polymeric nature of the glutenins by opposition to the 2 and 12 HMW-GS from Rubisko.

The same results can be observed with the other hydrodynamic parameters such as the number-average radii of gyration (R_n) and the weight-average radii of gyration (R_w).

Run	Box Behnken design ^(*)	Molecular features							
		UPP/EP	$M_n^{(a)}$ ($\times 10^5$) g.mol ⁻¹	$M_w^{(b)}$ ($\times 10^7$) g.mol ⁻¹	$R_n^{(c)}$ (nm)	$R_w^{(d)}$ (nm)	$v^{(e)}$	MP (%) (f)	[freeSH] ($\mu\text{mol/g}$ glutenin)
C	(-/-/-)	0.34	9.38	3.81	55.30	80.00	0.30	0.50	9.21
1	(0/-1/-1)	0.41	9.27	3.61	56.17	78.17	0.29	0.50	6.73
2	(+1/0/-1)	0.44	8.65	3.79	61.95	66.40	0.28	0.48	6.38
3	(0/+1/-1)	0.49	9.52	4.30	62.65	71.07	0.28	0.53	6.98
4	(-1/0/-1)	0.43	11.56	4.82	68.63	87.93	0.27	0.57	7.50
5	(-1/-1/0)	0.44	10.52	3.73	50.95	77.35	0.29	0.54	6.71
6	(+1/-1/0)	0.55	10.38	3.70	58.86	80.40	0.29	0.53	5.87
7	(+1/+1/0)	0.63	12.68	5.92	68.62	95.90	0.27	0.61	5.80
8	(-1/+1/0)	0.48	12.56	4.44	71.25	88.25	0.27	0.61	5.95
9	(0/-1/+1)	0.51	12.14	4.30	62.56	78.56	0.27	0.60	6.14
10	(+1/0/+1)	0.71	14.71	4.55	79.00	96.66	0.28	0.66	5.30
11	(0/+1/+1)	0.62	11.40	4.73	71.20	83.20	0.27	0.57	5.38
12	(-1/0/+1)	0.48	12.44	3.59	66.23	82.76	0.26	0.59	6.06
13	(0/0/0)	0.52	10.15	3.62	58.30	80.15	0.27	0.53	6.20
14	(0/0/0)	0.57	12.26	4.07	72.33	88.43	0.26	0.58	6.18
15	(0/0/0)	0.56	11.83	4.42	65.33	87.93	0.24	0.57	6.35
17	(+1/+1/+1)	0.58	15.34	6.77	72.36	98.40	0.25	0.66	5.89
18	(-1/-1/-1)	0.41	10.37	4.45	62.60	90.40	0.24	0.52	7.88

(*) Variable codification levels: (-1, 0, +1) for X_1 (2, 4, 6%), X_2 (60, 120, 180g.m⁻³), X_3 (15, 37.5, 60min) respectively, (a)Number-average molar mass, (b)Weight-average molar mass, (c)Number-average radii of gyration, (d)Weight-average radii of gyration, (e)Conformation plot slope, (f)MacroPolymer: polymeric proteins with a molar mass greater than $1 \times 10^6 \text{ (g.mol}^{-1})$.

Table I. Macromolecular features of polymeric proteins of Rubisko.

In table I, v represents the dependence of size upon mass for total glutenin polymers of Rubisko (i.e. value obtained by fitting the linear relation of the logarithmic form of $R=KM^v$). This linear relation can be considered as an estimation of the molecular conformation of the studied polymers. As it can be seen in table I, ozonation leads to a decrease of this hydrodynamic parameter v (from 0.30 to 0.25 for the control and the run17 respectively). This decrease indicates that molecular dimensions increased only slowly with the molecular weight and thus the molecules have a more compact structure.

SDS-PAGE profiles of wheat protein and free SH-group content

Both non-reduced (Fig. 1A) and reduced (Fig. 1B) SDS-PAGE were used to analyze the protein pattern changes in wheat flour as exposure to ozone gas. Proteins under non-reducing condition did not change remarkably due to the ozone treatment (Fig. 1A). Polymerization (bands with a $M_w \geq 250 \text{ kDa}$ on the top of the gel) seems to occur mainly through disulphide bonds, as evidenced by the reducing protein patterns shown in Fig. 1B, in which there was no major difference in the most of the visible bands among all samples. On the other hand, data in table I also showed that the free SH-group content in polymeric proteins decreased significantly as ozone treatment time increased indicating a potential formation of disulfide bonds in these proteins. On the top of the reduced separated gel (Fig. 1B), visibly increases in intensities of bands from the ozone treated samples

were also observed. That is, high molecular mass aggregates were formed even after the reduction of DTT. It can be suggested that the cross-linking was not occurring only through disulphide bonds. According to Linlaud et al.^[13], some non-covalent interactions may contribute to these aggregates.

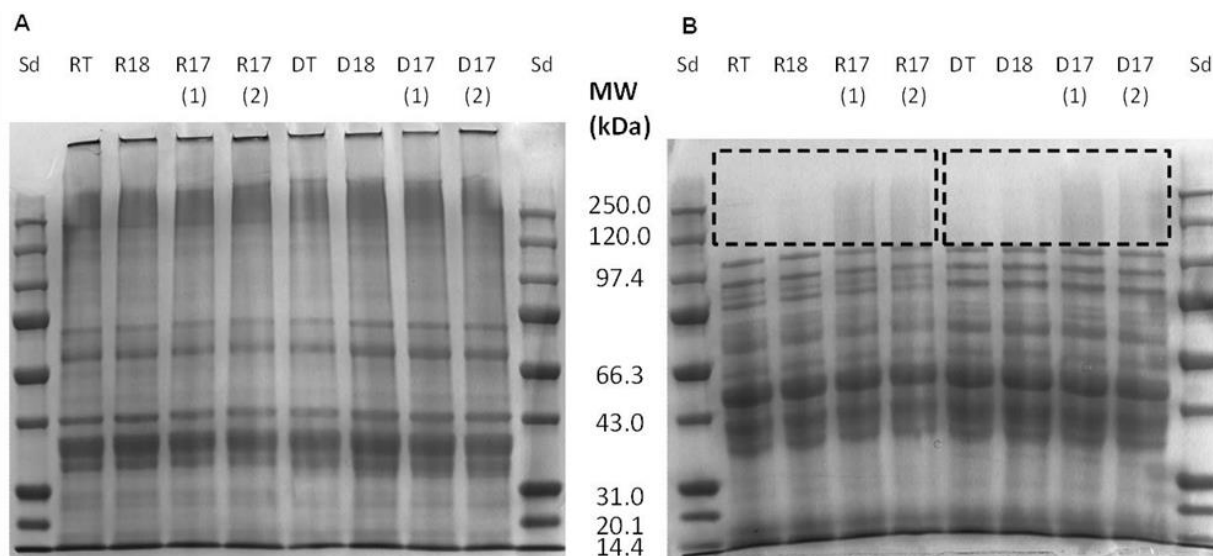


Figure 1. SDS-PAGE analysis of total protein patterns in flour of two bread wheat cultivars [(R) Rubisko and, (D) Diamento]. (A) the non-reduced patterns and, (B) the reduced patterns. Lanes 18(1), 18(2), 17(1) and, 17(2) corresponding to different ozone treatments [coded values (+1/+1/+1) respectively]. (1) and (2) replicate. (Sd) Molecular weight standards. (T) control (without ozone treatment).

Rheological properties of wheat proteins

As it can be seen in table II, there is a close relationship between the major alveographic parameters (i.e. W and P/L) and the O₃ consumption during the different gas treatments.

Run	Box Benhken design	Diamento				Rubisko					
		O ₃ consumption (gO ₃ /kg wheat grain)	W ^a (10 ⁻⁴ J)	P ^a (mm)	L ^a (mm)	P/L ^a	O ₃ consumption (gO ₃ /kg wheat grain)	W ^a (10 ⁻⁴ J)	P ^a (mm)	L ^a (mm)	P/L ^a
C	(-/-/-)	0.00	169	89	47	1.89	0.00	114	55	66	0.83
1	(0/-1/-1)	2.16	135	97	31	3.13	1.91	109	59	52	1.13
2	(+1/0/-1)	4.01	135	99	31	3.19	3.91	103	52	55	0.95
3	(0/+1/-1)	4.97	104	96	24	4.00	5.40	106	61	45	1.36
4	(-1/0/-1)	2.57	158	104	34	3.06	2.86	100	58	49	1.18
5	(-1/-1/0)	2.79	134	123	24	5.12	3.12	120	73	45	1.62
6	(+1/-1/0)	4.84	105	105	23	4.57	5.18	108	61	45	1.36
7	(+1/+1/0)	11.12	92	92	24	3.83	11.50	88	76	26	2.92
8	(-1/+1/0)	6.73	126	124	24	5.17	6.38	107	73	38	1.92
9	(0/-1/+1)	6.28	95	104	21	4.95	6.65	104	83	29	2.86
10	(+1/0/+1)	12.28	76	90	20	4.50	12.47	76	80	23	3.48
11	(0/+1/+1)	14.51	92	99	23	4.30	13.62	99	97	23	4.22
12	(-1/0/+1)	6.49	109	119	21	5.67	6.06	91	77	28	2.75
13	(0/0/0)	7.87	109	105	27	3.89	7.84	126	74	44	1.68
14	(0/0/0)	7.97	108	107	24	4.46	7.76	120	87	33	2.64
15	(0/0/0)	7.54	80	74	25	2.96	7.13	108	77	33	2.33
17 ^b	(+1/+1/+1)	16.11	63	80	19	4.21	18.09	58	69	21	3.29
18 ^b	(-1/-1/-1)	1.42	153	104	33	3.15	1.65	115	67	49	1.37
r ^c		1.00	-0.866			0.605	1.00	-0.718			0.859

^a Mean value of 5 experiments.

^b Extra runs.

^c Regression coefficient - significant up to 5% level.

Table II. Modifications of the rheological properties of wheat flours as a consequence of grain ozonation.

Whatever the wheat cultivar studied, all flours from ozonated wheat grains have W values lower than W control ($169 \times 10^4 \text{J}$ and $114 \times 10^4 \text{J}$ for Diamento and Rubisko respectively) and these values are negatively correlated ($r = -0.866$ and -0.718 for Diamento and Rubisko respectively) with the quantity of O_3 consumed by the wheat grains.

In the same time, as shown in table II, P/L of flours from ozonated wheat grains have higher values than control ($P/L = 1.89$ and 0.83 for Diamento and Rubisko respectively) and these values are positively correlated ($r = +0.605$ and $+0.859$ for Diamento and Rubisko respectively) with the quantity of O_3 consumed by the wheat grains.

All these results clearly showed that ozone treatment leads to flour with higher tenacity and lower extensibility than control wheat which can be easily explained by the increase of the amount of MacroPolymer (MP in table I). These polymers, mainly formed by the oxidation of free sulfhydryl groups to new disulfide bonds^[12], are characterized by more compact structure (i.e. lower v) resulting of an increase in protein-protein interactions.

Ozonation kinetics of molecular markers of wheat grain tissues

To follow the kinetics of penetration of O_3 within the whole wheat grain, we have measured and compared the oxidation of biochemical markers of (i) the peripheric layers (i.e. ferulic acid^[14]) and, (ii) the starchy endosperm (i.e. glutenins) (Fig. 2).

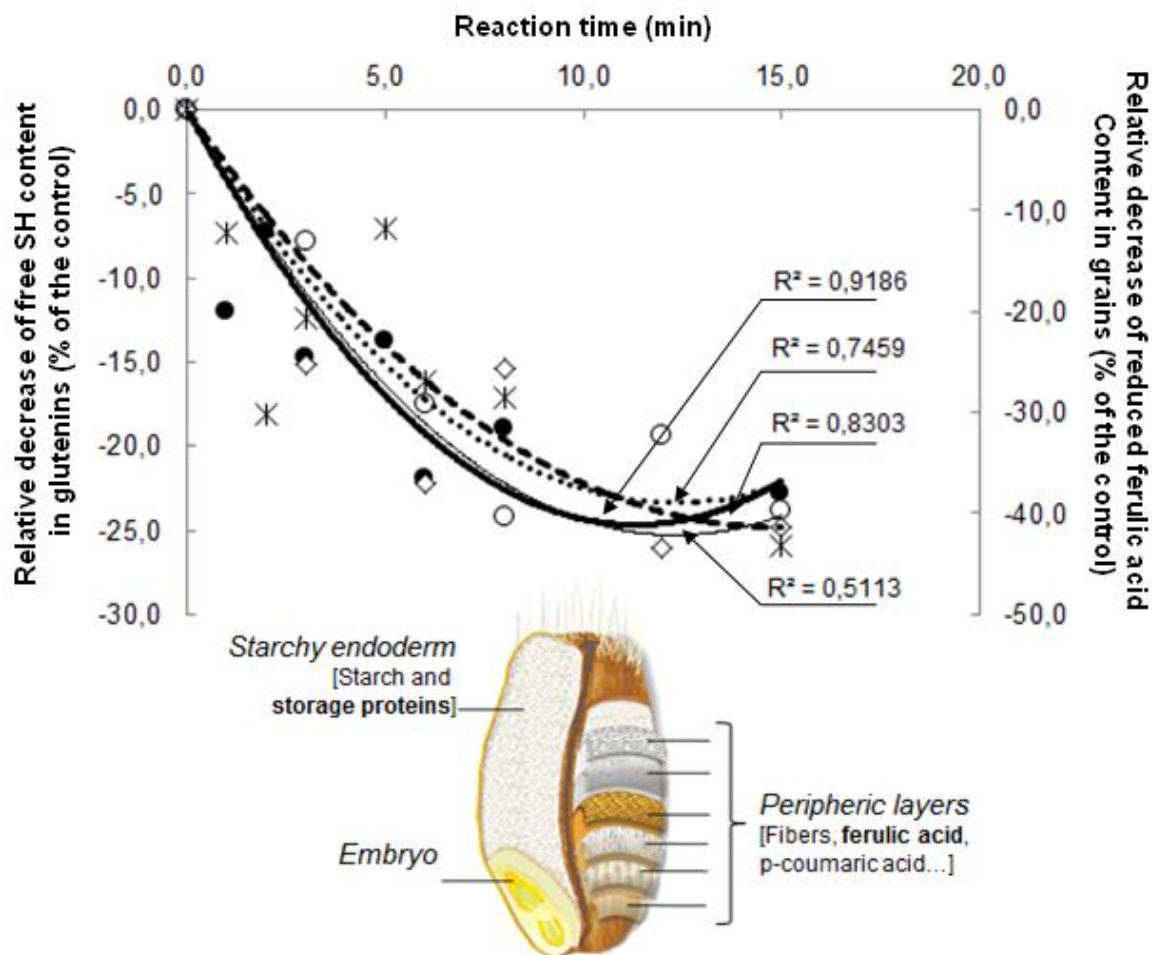


Figure 2. Oxidation of ferulic acid and glutenins present respectively in the peripheric layers and starchy endosperm of grain of the wheat cultivar Rubisko. Results are expressed in relative way (% of the control). Glutenin oxidation at different inlet ozone concentrations (.....) 60, (---) 120 and, (—) 180 $\text{gO}_3 \cdot \text{m}^{-3}$. Ferulic acid oxidation (—) at 180 $\text{gO}_3 \cdot \text{m}^{-3}$. Humidification rate was fixed at 4%.

As it can be seen in figure 2, the penetration of O_3 within the different grain tissues is very rapid (i.e. oxidation maximum obtained in less than 8-10 min) and without any O_3 concentration dependence. Furthermore, these results show that no significant difference can be seen between

the oxidation kinetics of the different markers used (i.e. ferulic acid and glutenins) demonstrating that wheat grain have an important porosity for ozone gas and this, in spite of, the important compartmentalized structure of wheat grain matrix.

Conclusions

Results showed that ozone treatment significantly reduced the SDS-solubility of the grain storage proteins which have an important effect on bread-making quality of wheat flour (i.e. increase in tenacity and decrease in extensibility of dough). The decrease of the SDS-solubility results from important modifications of the molecular weight distribution of polymeric proteins consecutive to ozone treatment of wheat grains. Furthermore, concomitantly to the increase of the molecular mass, ozonation leads to an increase of the molecular density of these polymers which can modify their SDS-solubility by reducing the SDS accessibility to the hydrophobic sites of these proteins. Even if, the oxidation of free sulfhydryl groups to new disulfide bonds can be advanced to explain all the molecular properties modifications of ozonated wheat grain proteins, we do not have to exclude the formation of some non-covalent interactions as suggested by our first results. Some authors^[15] suggested that dityrosine, which can be formed by tyrosine oxidation by ozone^[16], could be a new kind of stabilizing cross-link in the wheat gluten structure in addition to disulphide bonds. This hypothesis will be investigated in subsequent studies.

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