Food and Chemical Toxicology 101 (2017) 105–113

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Microwave-based treatments of wheat kernels do not abolish gluten epitopes implicated in celiac disease

Carmen Gianfrani [a, 1], Gianfranco Mamone [b, 1], Barbara la Gatta [c], Alessandra Camarca [b], Luigia Di Stasio [b, d], Francesco Maurano [b], Stefania Picascia [a], Vito Capozzi [e], Giuseppe Perna [e], Gianluca Picariello [b], Aldo Di Luccia [c, *]

[a] Institute of Protein Biochemistry, CNR, Naples, Italy
[b] Institute of Food Sciences, CNR Avellino, Italy
[c] Department. of the Sciences of Agriculture, Food and Environment, University of Foggia, Italy
[d] Department of Agriculture, University of Naples, Portici (Na), Italy
[e] Department of Clinical and Experimental Medicine, University of Foggia, Foggia (Italy) and National Institute of Nuclear Physics, Section of Bari, Italy

ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form
9 January 2017
Accepted 11 January 2017
Available online 12 January 2017

Keywords:
Microwave treatment
R5 and G12 ELISA
Gluten
Celiac disease
T cell assay
Mass spectrometry-based proteomics

ABSTRACT

Microwave based treatment (MWT) of wet wheat kernels induced a striking reduction of gluten, up to <20 ppm as determined by R5-antibodybased ELISA, so that wheat could be labeled as gluten-free. In contrast, analysis of gluten peptides by G12 antibody-based ELISA, mass spectrometry-based proteomics and in vitro assay with T cells of celiac subjects, indicated no difference of antigenicity before and after MWT. SDS-PAGE analysis and Raman spectroscopy demonstrated that MWT simply induced conformational modifications, reducing alcohol solubility of gliadins and altering the access of R5-antibody to the gluten epitopes. Thus, MWT neither destroys gluten nor modifies chemically the toxic epitopes, contradicting the preliminary claims that MWT of wheat kernels detoxifies gluten. This study provides evidence that R5-antibody ELISA alone is not effective to determine gluten in thermally treated wheat products. Gluten epitopes in processed wheat should be monitored using strategies based on combined immunoassays with T cells from celiacs, G12-antibody ELISA after proteolysis and proper molecular characterization.

1. Introduction

Wheat gluten proteins play an extremely important role in determining the quality of baked-foods, due to their ability to form a cohesive mass in dough once the flour is mixed with water (Bonomi et al., 2013). On the other hand, wheat proteins induce "*gluten-related disorders*", a term referred to all conditions related to food intake of gluten-based products, including celiac disease (CD), dermatitis herpetiformis, gluten ataxia, and non-celiac gluten sensitivity (NCGS) (Sapone et al., 2012). CD is an immune-mediated systemic disorder elicited by the ingestion of gluten-containing cereals (*i.e.* wheat, barley, rye). Prevalence of CD ranges between 0.5 and 1% over the general population. Etiopathogenesis of CD involves a complex series of genetic and environmental factors (Abadie et al., 2011; Sollid, 2000; Lundin and Sollid, 2014). CD is strictly associated with the HLA class II genes encoding for DQ2.5 and/or DQ8 molecules, as the majority of individuals with CD (>97%) carry either the DQ2.5 or DQ8 genes. The HLA DQ2.5/DQ8 molecules have a key role in CD pathogenesis, as they present immunogenic gluten peptide sequences to gluten-sensitive $CD4^+$ T lymphocytes (Abadie et al., 2011; Sollid et al., 2012). Tissue transglutaminase (tTG)-mediated deamidation of specific glutamine residues to glutamic acid increases the affinity of gluten peptides to the HLA molecules (Molberg et al., 1998; van de Wal et al., 1998).

Gluten proteins are classified into gliadins and glutenins, which account for nearly 80% of the total protein content of wheat kernel (Wieser, 2007; Bonomi et al., 2013). Because of the high percentage of proline residues, gluten proteins are resistant to gastrointestinal

Abbreviations used: AMBIC, ammonium bicarbonate; CD, celiac disease; IFN-γ, interferon gamma; MWT, microwave based treatment; NCGS, non-celiac gluten sensitivity; tTG, tissue transglutaminase; TCL, T cell line.

* Corresponding author. Dipartimento di Scienze Agrarie degli Alimenti e dell'Ambiente (DiSAAA), Università degli Studi di Foggia, Via Napoli, 25, 71122 Foggia, Italy.
E-mail address: aldo.diluccia@unifg.it (A. Di Luccia).

[1] The authors CG and GM share cofirst authorship.

http://dx.doi.org/10.1016/j.fct.2017.01.010
0278-6915/

digestion, so that large gluten fragments can reach the gut lumen at a high concentration, eliciting adverse immune responses in predisposing individuals (Hausch et al., 2002).

Numerous strategies have been developed for preventing or reducing gluten toxicity of wheat flour, as alternatives to a strict gluten-free diet of CD patients. The most interesting approaches are based on proteolytic degradation of gliadin epitopes or chemical modification of target residues of transglutaminase (Rizzello et al., 2007; Wolf et al., 2015; Gianfrani et al., 2007; Wieser and Koehler, 2012; Ribeiro et al., 2015).

Physical treatments of wheat using microwave (Leszczynska et al., 2003; Lamacchia et al., 2016) or pulsed light (Panozzo et al., 2016) irradiation have been recently proposed to reduce the immunoreactivity of gluten proteins. In these cases, the reduction of gluten immunoreactivity has been typically assessed by sandwich ELISA (*e. g.* R5-antibody ELISA).

In the present study, we investigated the chemical and structural modification induced by microwave based treatment (MWT) of wet wheat kernels on gluten proteins. The effect of MWT on either gliadin extracts or peptides derived from enzymatic hydrolysis (pepsin-trypsin or chymotrypsin) of gluten were investigated using R5 and G12 ELISA, *in vitro* assays with T cell from gut mucosa of celiac subjects, Raman spectroscopy and mass spectrometry-based proteomics.

2. Materials and methods

2.1. Chemicals

Enzymes (pepsin, trypsin, chymotrypsin, tTG), Tris-HCL, ammonium bicarbonate (AMBIC), KCl, EDTA and HPLC-MS grade solvents were all provided by Sigma Aldrich (Italy). T-cell culture medium and supplement were from Lonza-BioWhittaker (Verviers, Belgium). The interferon (IFN)-γ antibodies from MabTech (Nacka Strand Sweden).

2.2. Microwave-based treatment

Kernels from *T. durum* (Ofanto cultivar) and *T. aestivum* (Centauro cultivar) were furnished by Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA-QCE) and Borrelli farm (Foggia, Italy), respectively. Different MWT of wheat kernels were performed. Briefly, wheat kernels (100 g) were soaked in tap water (150 or 500 mL), and then irradiated in a commercial MW oven (Whirlpool Corporation, Benton Harbor, Michigan, US). Combinations of MW oven power, wetting water volume, wetting and drying time are detailed in Table 1. The granulometry of milled treated and untreated kernels were measured by Horiba laser granulometer LA-950 (Retsch Technology GmbH, Haan, Germany).

2.3. Raman spectroscopy

Raman spectra of flour samples were obtained with a micro-Raman spectrometer (LabRam, Jobin-Yvon Horiba), operating at room temperature by using a He-Ne laser ($\lambda = 632.8$ nm). The laser beam was focused on samples by an Olympus optical microscope (LMPlanFl, Olympus). A laser power of 3.5 mW was measured at the sample position, in order to obtain an acceptable signal/noise ratio, but avoiding thermal damage of the sample. Each Raman spectrum was measured in the 1550 to 1725 cm^{-1} range from, with an integration time of 10 s averaged over 15 accumulations. Scattered light was collected in backscattering geometry and a notch filter was used to suppress the elastic component of the scattered radiation. The collected light beam was dispersed into a spectrophotometer equipped with a 600 grooves/mm grating. The spectral resolution was ~3 cm^{-1} per pixel. A cooled charge coupled device (CCD) system, cooled at 223 K, detected the Raman spectrum, and a separate CCD camera collected white light microscope images of the sample being probed. For each flour sample, 20 different points were randomly selected and investigated. Spectra were processed by PeakFit 4.12, Systat Software.

2.4. Extraction and enzymatic hydrolysis of gliadin

Alcohol soluble proteins (gliadin) from milled MWT and untreated (control) kernels were extracted according to the classical fractionation procedure (Wieser et al., 1998). Gliadin aliquots were analyzed by SDS-PAGE. Pepsin and trypsin (PT) digests of gliadins were prepared according to Gianfrani et al. (2007).

2.5. Digestion of gluten proteins

Unextracted gluten proteins from milled MWT and untreated (control) kernels were digested by chymotrypsin. To this end, milled kernels (100 mg) were depleted of the saline buffer soluble protein fraction (albumins and globulins) with 100 mM KCl, 50 mM Tris-HCl, pH 7.8, 5 mM EDTA in a rotary shaker for 10 min at room temperature (twice). After centrifugation at 10.000 rpm for 15 min, the pellet was re-suspended in 100 mM AMBIC, at pH 7.8 and incubated overnight at 37 °C in a thermomixer with chymotrypsin (1:50 enzyme:substrate, w/w ratio). Sample was then centrifuged at 10.000 rpm for 20 min. Supernatant was collected and stored at −80 °C until further analysis. Prior to T cell stimulation assays, gliadin/gluten digests were deamidated by tTG as reported previously (Gianfrani et al., 2007).

2.6. Evaluation of gluten content by R5 sandwich and G12 competitive ELISA

Gluten content of MWT wheat kernels was measured by R5 sandwich ELISA, using a Ridascreen® Gliadin Elisa Kit (R-Biopharm AG Darmstadt, Germany) according to the manufacturer's and to

Table 1
Chemical and physical parameter soft microwave treatment of whole wheat kernels.

Treatment	Kernels (g)	Water (mL)	Time in water (min)	External drying	MW (W)	Time in microwave (min)	Granulometry μm	Milling	R5-ELISA (ppm)	G12-ELISA (ppm)
Control	100	/	/	/	/	/	<529	immediately	>600	>600
MWT	100	500	1min	RT	1000	2	<185	After 24 h	70 ± 10	>600
MWT0	100	500	2 h	drained	1000	2	<200	After 24 h	41 ± 8	>600
MWT1	100	150	1 h	damp	600	5	<196	immediately	29 ± 5	>600
MWT2	100	150	1 h	damp	600	3	<584	immediately	42 ± 8	>600
MWT3	100	150	1 h	wet	600	3	<269	immediately	42 ± 8	>600
MWT4	100	150	1 h	damp	600	6	<185	immediately	<5	>600