Food Research International 91 (2017) 92–102

Contents lists available at ScienceDirect

Food Research International

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

Peptides from gluten digestion: A comparison between old and modern wheat varieties

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ARTICLE INFO

Article history:
Received 3 October 2016
Received in revised form 23 November 2016
Accepted 27 November 2016
Available online 2 December 2016

Keywords:
Wheat breeding
Coeliac disease
Gluten peptides
In vitro digestion
Old wheat

ABSTRACT

Coeliac disease is an autoimmune enteropathy that develops in genetically predisposed subjects after the ingestion of gluten or related proteins. Coeliac disease has an increasing incidence in the last years in western countries and it has been suggested that wheat breeding might have contributed to select more toxic forms of gluten. In this work, we analysed gluten peptides generated by *in vitro* digestion of different old and modern *Triticum* varieties, using LC-MS. We concluded that old varieties analysed produced a higher quantity of peptides containing immunogenic and toxic sequences than modern ones. Thus old wheat lines are not to be considered "safer" for subjects that are genetically predisposed to celiac disease.

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1. Introduction

Old cereal species and varieties have been widely promoted in the last years in order to exploit their characteristic organoleptic properties, the (real or alleged) nutritional properties and the intrinsic cultural and historic value. These species and varieties are increasingly in demand by consumers and the agro-food industry is strongly investing in the study of innovative products based on these cereals. Indeed, several studies focused on the phenolic components of old common wheat lines, finding good basis for antioxidant and chemo protective properties (Leoncini et al., 2012). Old wheat varieties contain indeed a wider spectrum of phenolic compounds, derived from the higher genetic biodiversity, compared to the modern one (Dinelli et al., 2011), even if the content of health beneficial substances can be influenced by the sowing season (Rascio et al., 2015). Other studies on durum wheat found good prebiotic activity of soluble fibre extracted from old durum-type wheat grains, so they could be used as a source for prebiotic formulations (Marotti et al., 2012). An extensive database of phytochemicals and dietary fibres was done comparing landraces and modern wheat varieties, demonstrating that wheat breeding did not affect these wheat components (Shewry et al., 2011). Other studies demonstrated that ancient wheat is not healthier than modern wheat, in terms of bioactive compounds present (Shewry & Hey, 2015). However, old wheat varieties are more adaptable to adverse environments. Thus, they could represent a solution for local communities, were the commonly grown wheat varieties are not cultivable (Migliorini et al., 2016).

In the last years, the increasing incidence of coeliac disease has shifted the focus also to the protein fraction of old wheat varieties. Coeliac disease is an autoimmune enteropathy that develops in genetically predisposed subjects after the ingestion of gluten or related proteins of barley (hordeins) and rye (secalins) (Kagnoff, 2007). The possibility to eat oats by celiac patients is still debated, since different oat varieties showed different activation of the gluten-induced transglutaminase-2 (TG2)-dependent events (Silano et al., 2014). During gluten digestion, some resistant peptides are formed due to the high proline content of prolamins (Hausch, Shan, Santiago, Gray, & Khosla, 2002); some of these peptides contain sequences (epitopes) able to trigger the immunological reaction in coeliac patients. Beside the immunological implications, gluten is very important also for the technological point of view, because it determines the viscoelasticity of the dough in bread making and in the production of wheat derived products (Goesaert et al., 2005). The varietal selection undertaken by breeders in the last decades in order to achieve the desired rheological properties has led to a decrease in the genetic biodiversity of wheat varieties present nowadays on the market (Fu & Somers, 2009). So, the higher biodiversity of old wheat lines was studied in order to identify genotypes encoding a lower number of coeliac disease epitopes (van den Broeck et al., 2010). However, T-cell proliferation assays demonstrated that all strains of wheat, independent of ploidy or old/modern origin, triggered heterogeneous responses covering wide ranges of stimulation indices. So,

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http://dx.doi.org/10.1016/j.foodres.2016.11.034
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according to the reported data, old strains of wheat, although previously suggested to be low or devoid of coeliac toxicity, should be avoided by coeliac patient and should not be considered "safe".

Nevertheless, old varieties, even if dangerous for coeliac patients, could actually be producing, upon gastrointestinal digestion, less immunotoxic peptides, thus actually helping to prevent the spread of the disease among predisposed subjects. If a lower content of toxic peptides after digestion would be proven true for old varieties, it would indeed be a strong indication that modern varieties have somehow a role in the current increase of the coeliac disease. However, there are only few studies comparing old and modern *Triticum* varieties, representing a knowledge gap.

In this paper, we compared the peptide profile of different *Triticum* species (old and modern), generated after simulated gastrointestinal digestion. In this way, it was possible to evaluate differences in the peptide pattern generated from the *in vitro* digestion of the different species, with particular focus on peptides containing sequences known in literature to be implicated in coeliac disease.

2. Materials and methods

2.1. Samples

Triticum samples analysed are reported in Table 1, together with the protein content on dry matter. Protein content is expressed as a range (minimum and maximum content found), taking into account for each variety the three different harvesting conditions (Parma/organic, Parma/conventional, Bologna/conventional). All the samples were harvested in two different Italian regions (Parma and Bologna) in the conventional way. To investigate a possible effect of organic farming, in the Parma area conventional and organic farming were compared. Three biological replicates (harvested in three different independent blocks) were taken for all the samples. Samples were ground with an analytical mill (A11 basic, IKA, Staufen, Germany). Flour was not defatted prior to digestion.

2.2. Reagents and solvents

Deionised water was obtained from a Millipore Alpha Q-Waters purification system (Billerica, MA, USA). Pepsin from porcine gastric mucosa, trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas, α-amylase from barley malt (type VIIIa), uric acid, mucin from porcine stomach (type III), glucose, glucuronic acid, glucosamine hydrochloride, albumin bovine, pancreatin from porcine pancreas, lipase from porcine pancreas (type II), bile from bovine and ovine, sodium dihydrogen phosphate, potassium chloride, urea, acetonitrile, ethanol, Fmoc-glutamine(Trt)-OH, piperidine, diisopropylethylamine, dichloromethane, triisopropylsilane, tyrosine, phenylalanine, tris(hydroxymethyl)-aminomethane, urea, dithiothreitol and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37% V/V), sodium hydroxide, sodium hydrogen carbonate, calcium chloride, ammonium chloride and dimethylformamide were purchased from Carlo Erba (Milan, Italy). Fmoc-leucine-OH, Fmoc-proline-OH, HBTU and Fmoc-tyrosine(tBu)-Wang resin were purchased from Novabiochem (Darmstadt, Germany). Formic acid and trifluoroacetic acid were purchased from Acros Organics (Geel, Belgium). Sodium chloride was purchased from AnalaR Normapur (Milan, Italy). Potassium thiocyanate was purchased from Riedel De Haen (Seelze, Germany). Magnesium chloride was purchased from Merck (Darmstadt, Germany).

Table 1
Old and modern[a] wheat varieties studied.

Species	Variety	Type	Protein content (% on dry matter)
Triticum aestivum L.	Grano del Miracolo	Old	11.8–13.2
	Virgilio	Old	12.0–13.7
	Blasco	Modern	11.2–12.8
	Bologna	Modern	11.7–12.4
Triticum turgidum var. *durum* Desf.	Senatore Cappelli	Old	14.1–15.0
	Timilia	Old	12.3–13.9
	Odisseo	Modern	11.5–13.1
Triticum monococcum L. (einkorn)	ID331	Modern	13.3–17.3
Triticum turgidum var. *dicoccum* L. (emmer)	Farro della Garfagnana	Old	11.9–13.4
Triticum spelta L. (spelt)	Rouquin	Modern	12.4–13.7

Thus with the term "old" we refer to a variety already existing before the first world war, and with the term "modern" to a variety first developed after the first world war.
[a] The term "old" and "modern" in this context are considered as related to the heavy breeding developments which started after the first world war.

2.3. In vitro digestion

The samples were digested as described by Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005) with the volume of digestive juices scaled to the smallest amount of sample. Briefly, 450 mg of sample were incubated 5 min with 600 μL of saliva (ratio α-amylase:matrix 1:1300); after this phase, 2.4 mL of gastric juice (ratio pepsin:matrix 1:38) were added and the sample was incubated for 2 h. To set the pH for the intestinal phase, 400 μL $NaHCO_3$ 1 mol L^{-1} were added; after, 2.4 mL of duodenal juice (ratio pancreatin:matrix 1:10, ratio lipase:matrix 1:63) and 1.2 mL of bile (ratio bile:matrix 1:6) were added and the sample was incubated for 2 h. All the digestion steps were carried out at 37 °C. At the end of the digestion, 58.3 μL of HCl 37% were added to inactivate enzymes and the sample was centrifuged at 8965*g* at 4 °C for 45 min, in order to precipitate insoluble compounds and undigested proteins. No dialysis of the sample was performed. Prior to LC-MS analysis, all samples were filtered with a cut off of 0.45 μm. For the quantification, 295 μL of digested sample were spiked with 5 μL of standard peptide solution (LQLQPF(d_5)PQPQLPY, 0.477 mmol L^{-1}). Since in this digestion model also exopeptidases are used and different truncated of its analogue natural peptide are found in the digested sample, the internal standard was added at the end of the digestion (after enzyme inactivation) in order to avoid its digestion or degradation.

2.4. Synthesis of the internal standard

The internal standard was synthesized in our laboratory as previously described by Prandi et al. (2012). Briefly, the peptide LQLQPF(d_5)PQPQLPY was synthesized on solid phase according Fmoc/t-butyl strategy using a Syro I Fully Automated Peptide Synthesizer (Biotage, Uppsala, Sweden). The ring of phenylalanine was labeled with five deuterium atoms (Mr + 5). The peptide was cleaved from the Wang-resin using a trifluoroacetic acid with 2.5% of triisopropylsilane and 2.5% of water as scavengers. Peptide purification was carried out on a semipreparative RP-HPLC-UV (detection wavelength: 280 nm, where tyrosine absorbs). The separation was obtained using a Jupiter 5 μm, C18 300 Å column (250 × 10 mm, Phenomenex) using a gradient elution. Eluent A was water and eluent B was acetonitrile. Both A and B eluents were acidified with 0.1% of formic acid. The flow was 4 mL/min and the gradient was: 0–5 min 100% A, 5–15 min from 100% to 60% A, 15–25 min 60% A, 25–30 min from 60% A to 0% A, 30–35 min 0% A, 35–40 min from 0% to 100% A, and 40–55 min 100% A. The purified product was quantified with a spectrophotometer at a wavelength of 280 nm using an external calibration curve made with an equimolar solution of tyrosine and phenylalanine.

2.5. UPLC/ESI-MS analysis

UPLC/ESI-MS method for peptide separation and quantification was previously developed in our laboratory as described by Prandi et al.

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