Proceedings of the 32nd Meeting

WORKING GROUP
on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler

27 - 29 September 2018
Ayr, Scotland, United Kingdom

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Cover picture* and picture of participants
Thomas Mothes

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* Cover picture: Chandelier in the lecture room of Oswald Hall, venue of the 32nd PWG-meeting

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Preface

Already in the 2015 meeting of the Working Group on Prolamin Analysis and Toxicity (PWG) in Tulln, Austria, Pauline Titchener from Neogen Europe Ltd. informed me that Neogen would be willing to host one of the following PWG meetings in their European headquarter in Scotland. Finally, 2018 was the year that this became reality. The meeting was held at Oswald Hall, a historic building belonging to the Neogen campus in Ayrshire. The lecture room was very special with its historic interior decoration but also with modern facilities that are needed for a conference. Pauline and her team were present during the entire meeting. Pauline was also available after the official programme and organised a joint whisky tasting and dinner. Apart from the group members the audience comprised an invited speaker, guests from academia, industry, and international coeliac societies. Representatives from cereal starch producers, producers of gluten-free foods, as well as manufacturers of kits for gluten analysis and of kits for antibody tests in the serology of coeliac disease (CD) participated from industry.

Analytical and clinical work in the field of CD, non-coeliac gluten/wheat sensitivity (NCGS/NCWS), wheat allergy and gluten done in the labs of PWG members as well as results of guests and invited speakers were presented in 18 talks and lively discussed at the meeting. This time, legal and regulatory aspects of gluten analysis were not discussed because only a few participants from coeliac societies were able to attend the meeting. A symposium with presentations looking at the successful determination of the wheat genome and its exploitation in future research was organised and highly estimated by the audience.

I am grateful to all participants for their active contributions as presenters as well as during the discussions. This made the 2018 meeting a great success. I would like to express my special thanks to Neogen Europe Ltd., in particular to Pauline Titchener, for being a perfect host as well as to Sharon Forsythe for her help in the organisation of the meeting. Special thanks go to Thomas Mothes and Martin Stern for their long-time dedication to the group. Both left the group by the end of 2018. Finally, I express my gratitude to all friends, colleagues, sponsors and participants for their inspiration and continuing support of the PWG.

Esslingen, March 2019 Peter Koehler

Table of Contents

1 Executive summary

Eighteen presentations covered all aspects related to gluten, coeliac disease (CD) and other relevant hypersensitivities. Sixteen authors have sent manuscripts that are compiled in this proceedings book. Some coeliac societies were not able to send delegates because of a meeting of the Association of European Coeliac Societies (AOECS) at the same time. Therefore, legal aspects were not covered in this meeting.

Analytical session

Seven presentations covered the analysis of gluten and other proteins of interest for the PWG. It became obvious that ELISA is currently the method of choice for gluten quantitation because this method was used in five presentations. Apart from the comparison of G12 and R5 ELISAs, a new ELISA for total gluten using three monoclonal antibodies was introduced. The unsolved problem of suitable reference materials for gluten quantitation was covered in two talks. Finally, two studies were presented that used LC-MS. One presentation provided data on the concentrations of amylase-trypsin inhibitors (ATI) in different wheat species, and in another study, isopeptides between tissue transglutaminase and wheat gluten peptides were identified.

Clinical session

One of the six presentations provided evidence for reduced activity of Einkorn in CD because of low resistance of peptides to proteolytic cleavage. Another study showed that there appears to be a discrepancy between serology and inflammation in potential CD. The third presentation showed that there are several pathways of apoptosis in untreated CD. Studies on the kinetics and transcriptomic profile of antigen-specific cells after gluten challenge showed that sampling between day 6 and 8 after 3-day gluten challenge is an appropriate time window for collection of gluten-specific T cells. Finally, there is evidence that deamidated gliadins worsen immune reactions in wheat allergy. This is of practical relevance because some industrial processes use deamidation to functionalise gluten proteins.

Symposium: Wheat genomics

The symposium included two presentations of recognised experts in wheat genomics. In a very exciting talk, the latest results of genome sequencing were reported. These activities resulted in the first wheat reference genome, and it was discussed, how this novel knowledge can be exploited in the near future. The second presentation was also very interesting and dealt with the latest approaches on wheat genome editing by the CRISPR/Cas technology. The first results of a study on eliminating coeliac active epitopes in wheat were discussed. The symposium showed that genome editing and sequencing are currently among the leading scientific topics in cereal research.

Participants of the 32nd Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Ayr, Scotland, UK, 27. - 29. September 2018

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3 Programme

THURSDAY, 27 September 2018

19:00 Arrival of Prolamin Working Group and all participants
Informal get-together with dinner
Welcome by Pauline Titchener
Location: Mercure Ayr Hotel

FRIDAY, 28 September 2018

08:30 Bus transfer to the meeting venue

09:00 Opening of the meeting (Peter Koehler)

09:05 Presentation by Neogen
Pauline Titchener, Ayr, UK

09:20 Analytical research reports

- *Chirdo, Ciclitira, Koehler, Lundin, Mothes, Scherf, Schuppan, Smulders, Tranquet; guests*

10:40 Coffee break

11:10 Analytical research reports (continuation)

12:10 Clinical research reports

- *Catassi, Chirdo, Ciclitira, Lundin, Mothes, Schuppan, Tranquet; Troncone; guests*

12:50 Lunch

14:00 Clinical research reports (continuation)

15:50 Coffee break

15:50 The Prolamin Working Group Executive Meeting (members only)

16:50 Bus transfer to the hotel

18:00 Bus departure from the hotel for all participants for the joint dinner
Location: Robert Burns Museum
Murdoch's Lone, Alloway, Ayr ,KA7 4PQ

23:00 Bus transfer to the hotel

SATURDAY, 29 September 2018

08:30 Bus transfer to the meeting venue

SYMPOSIUM

09:00 Wheat Genomics
Chair: Thomas Mothes, Leipzig, Germany

09:05 The first reference genome sequence for bread wheat
Manuel Spannagl, Neuherberg, Germany

09:50 Gluten genomics and epitope interactions
René Smulders, Wageningen, The Netherlands

10:35 Coffee break

11:15 Discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry, and guests

Outline: action plan 2019 of the Prolamin Working Group

13:00 Lunch and farewell
Bus transfer to the hotel

Afternoon/evening

Extra time for informal meeting and additional Prolamin Working Group meeting concerning action plan (hotel lobby)

Whisky tasting and joint dinner

SUNDAY, 30 September 2018

Departure of the Prolamin Working Group

4 Analytical research reports

4.1 AOAC International update: Gluten in oats method validation framework

Paul Wehling, Harrison Feldkamp

General Mills, Inc., Minneapolis, MN, USA

Introduction

In September 2017, the AOAC International Stakeholder Panel for Alternative Methods (ISPAM) adopted Standard Method Performance Requirement (SMPR) 2017.021, Quantitation of Wheat, Rye, and Barley Gluten in Oats, as the guidance document for the validation of methods for measuring gluten in oat products [1].

In the past, gluten methods were evaluated for accuracy based on spiking wheat gluten into various gluten-free (GF) matrices and estimating recovery of the method by calculating the percentage of analyte recovered during a multi-lab collaborative study. Recent quantitative methods, such as AOAC OMA methods 2012.01 and 2014.03 have used this process. In the case of validating an ELISA method for gluten in oats, it will be essential to evaluate the kit responses to not only wheat, but also barley and rye. The SMPR 2017.021 has indicated that for this method project, the responses of wheat, rye and barley should be estimated independently as part of single-lab validation. In order to facilitate such validations, a series of samples were prepared, each spiked with a single grain at specific levels. The SMPR states that for approval as an OMA method, the candidate method must demonstrate recovery of wheat, rye and barley gluten proteins separately, and the recoveries must be between 50% and 200%. This represents a new approach to the validation of gluten methods, where historically only wheat proteins have been considered as relevant to method accuracy.

Tab. 1 shows current AOAC Official Methods of Analysis (OMA) which have been validated for analysis of gluten in foods. Recent practice within AOAC is to restrict approval of the method to matrices which were studied in single lab validation (SLV) and/or in multi-lab validations (MLV), such as with a collaborative study. There currently is no OMA method applicable to oat products. Oat products are unique in the gluten-free supply chain in that there is a significant probability of encountering low-level barley contamination from agricultural commingling due to geographical areas where oats and barley are grown contiguously, specifically in Northern United States, and Western Canada. In the past 10 years, oat processors have developed systems to produce GF oats, either by mechanical/optical separation, or by selective growing/IP agricultural processes. In both of these systems, barley and wheat contamination are the most common sources of gluten containing grains. As such, it is critical that a

method being used to inspect and control a GF oat process be accurate to both wheat and barley proteins.

In the past, most of the focus of calibrating and validating gluten methods was on accuracy to wheat proteins. The Skerritt antibody was developed originally to measure wheat and has very low response to barley proteins [2]. The R5 antibody was raised against rye proteins and has reported high response to barley proteins [3]. Both methods were successfully validated with acceptable recovery of wheat gluten proteins.

Table 1. *Current AOAC International Official Methods of Analysis (OMA) for gluten*

OMA No.	Antibody	Action	Matrices	Comment
991.09	Skerritt	Final	"Foods"	Very low barley response
2012.01	R5	Final (2016)	Rice and Corn	Very high barley response
2014.03	G12	First (2014)	Rice Flour and Rice products	Some reported oat cross-reactivity
2015.05	R5	First (2015)	Fermented Cereals	Competitive Assay
2015.16	R5	First (2015)	Corn	LFD Qualitative

LFD, lateral flow device

Materials and methods

In order to validate the response of a method to wheat, rye and barley proteins separately, AOAC has produced a series of reference materials. This series consists of seven spiked samples, which are made from GF oat flour, quantitatively spiked with various levels of wheat, rye and barley flours. Tab. 2 shows the spike levels of each of the seven materials.

Table 2. *AOAC International reference samples for gluten validation*

Sample Name	Contaminant Grain	Level (as gluten)	Diluent Grain
Blank	None	0 mg/kg	Oat Flour
W10	Wheat	10 mg/kg	Oat Flour
W20	Wheat	20 mg/kg	Oat Flour
R10	Rye	10 mg/kg	Oat Flour
R20	Rye	20 mg/kg	Oat Flour
B10	Barley	10 mg/kg	Oat Flour
B20	Barley	20 mg/kg	Oat Flour

Spiking materials

GF Oats were obtained from General Mills, Inc., USA, by optical and mechanical sorting, dehulling and further optical sorting of dehulled groats. Oat groats were then milled with a Retsch Mill ZM200 to obtain oat flour, which tested at <1 mg/kg by the R5 method by replicate analysis (mean of 18 reps at 5 g test portion).

For rye and barley spike materials, blends were made for each grain from several samples of selected grain cultivars obtained from seed breeders in the region. In the case of rye, eight separate cultivars were blended in equal parts, then milled to flour to obtain the blended spike material. For barley, six cultivars of 2-row barley, plus three cultivars of 6-row barley were milled, then blended together to obtain a spiking flour.

For wheat, we were unable to obtain pure cultivars, so we instead used a mixture of commercially available whole-wheat flours, and flours made from commercially obtained wheat samples. In all, ten samples of wheats and whole wheat flours were blended to make a spike flour representative of North American wheats grown in 2015-2017.

Characterisation of Spiking Materials

The three spiking flour blends were analysed for total protein by Dumas (N x 5.83) nitrogen method. In order to estimate the level of gluten in each of the three spiking blends, the AOAC Working Group approved the use of a wet chemical extraction method to extract off non-gluten proteins and analyse the remaining solid pellet by Dumas nitrogen and compare to the unextracted protein level. This extraction method was based on the Codex Alimentarius definition of gluten as “the protein fraction from wheat, rye or barley to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl.” [4]. The following method was used to estimate gluten levels in the spiking materials.

1. Mill the grains through Retsch Mill ZM 200 with 0.5mm screen.
2. Weigh 150 mg sample grain into a 2 mL microcentrifuge tube. Record the weight to the nearest 0.1 mg.
3. Add 1.5 mL water to the tube. Cap and vortex to completely disperse the sample.
4. Let the sample stand at ambient temp for 15 min, vortexing every 5 min.
5. Centrifuge in micro centrifuge for 10 min at 3400 RPM.
6. Decant off the supernatant, making sure not to lose any solids. If solids are not completely at bottom of the tube, recentrifuge an additional 10 min.
7. Repeat steps 3-6 with water.
8. Repeat steps 3-6, 2 times with 0.5 M NaCl/PBS solution.
9. Place the tube in vacuum oven and dry overnight at 70 C under vacuum for 16 hours.
10. Remove from vacuum oven, put pellet in Dumas foil and drop in furnace to measure nitrogen content. Use original flour weight as mass for Dumas calculation
11. Report N2 content per sample weight of original sample before washing.
12. Compare N2 content vs Dumas reading with no solvent treatment.
13. If needed, report % protein as %N x 5.83

Results and discussion

The three samples of flour spiking materials were analysed five times by Dumas protein and five times by the wet chemical method above. Tab. 3 gives results of the characterisation.

Table 3. *Observed gluten levels of spiking materials*

Blend	Total Protein (g/100 g)	Gluten remaining wet chem. (g/100 g)	Fraction wet chem./total
Rye	7.82	4.04	0.52
Barley	10.17	7.93	0.78
Wheat	12.46	9.21	0.74

The dilution and manufacturing of the reference materials was performed by Trilogy Labs, Washington, MO, USA to produce the series of seven samples as given in Tab. 2. The Materials are available for purchase through United States Pharmacopeia, Rockville MD, USA, (Cat. No. 1294839).

As a demonstration of the suitability of the materials and in order to provide an example of the process for estimating recovery, we have analysed each of the seven samples with replication (18 replicates at 5 g test portion level) by the R5 method (R-Biopharm kit R7001) and report the results as follows in Tab. 4. Figure 1 is a plot of these data.

Table 4. *Observed gluten levels of reference materials by R5 antibody*

Reference Material	Nominal Level (mg/kg)	Mean Gluten (mg/kg)	Std Deviation (mg/kg)	95% Confidence interval of mean
Blank	0	0.819	0.463	(0.61, 1.03)
Rye10	10	48.2	3.13	(46.7, 49.6)
Rye20	20	85.9	4.93	(83.6, 88.2)
Wheat10	10	14.6	5.20	(12.2, 17.0)
Wheat20	20	25.1	2.68	(23.9, 26.4)
Barley10	10	38.0	4.01	(36.1, 39.9)
Barley20	20	70.6	4.70	(68.4, 72.8)

Figure 1. *Plot of response for R5 kit to spiked recovery samples*

To estimate overall recovery, we recommend plotting all individual replicates as observed results vs. spiked nominal value and regressing as linear model ordinary least-squares regression. The slope of the regression line will be the recovery estimate. Tab. 5 is a summary of the recovery data obtained for the three grains.

Table 5. *Statistical summary of R5 kit response plot*

Grain	Slope	Intercept (mg/kg)	Recovery (%)	95% CI on recovery (%)
Wheat	1.22	1.36	122	110 – 133
Rye	4.25	2.42	425	412 – 439
Barley	3.49	1.58	349	337 – 361

Recovery = slope x 100,
95% Confidence interval based on z-distribution of slope given slope and standard error of slope estimate from regression of all individual points:
CI = (slope ± 1.96 SE) x 100

Conclusions

This new AOAC protocol represents a new approach to validating gluten methods. While in the past, emphasis was put on accuracy with respect to wheat proteins, here we are looking as well at trying to achieve balanced response by all three possible gluten sources.

The AOAC SMPR requires a proposed method to demonstrate recoveries on all three grains between 50 and 200%. By the analysis shown here, the R5 antibodies, in conjunction with the current Mendez Cocktail extraction (AOAC OMA Method 2012.01) would not be considered suitable for use in oat products due to its high responses for barley and rye (349% and 425% recovery, respectively). This over-response is higher than reported in prior reports [3] where the recovery was estimated to be around 200% with the PWG gliadin calibrators and 2 x gliadin correction factor. The estimates in that reference were made based on extracted proteins in solution, not against grains spiked into grains, then extracted by a set method protocol. We feel the results given here are better estimates for the recovery of the complete method, including extraction. It is very important when estimating method recovery to include the extraction steps from the method under study in the experiment. We have observed very different recoveries on these reference materials with different extraction methods, even with the same antibody system.

In addition, it should be noted that these reference materials are spiked samples, and the contaminant grains have been milled prior to spiking. This makes the repeatability of the methods very tight as opposed to incurred samples, where the particle sizes of the contaminant grains are larger. We recommend that these reference materials be used only for recovery studies, and not for precision estimates, as the precision estimates on these reference materials will be much lower than observed on naturally incurred oat flour samples. Precision studies should only be carried out with incurred samples of the specific matrices under study.

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4.2 RIDASCREEN® Total Gluten R7041

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Introduction

The RIDASCREEN® Gliadin R7001 from R-Biopharm AG is based on the monoclonal R5 antibody and has been endorsed as Codex Alimentarius Type I method, AOAC Official Method™ of Analysis 2012.01 Final Action and AACCI approved method 38-50.01 [1-3]. The main epitope of the R5 antibody is the pentapeptide QQPFP [4], which is present in many replicates in prolamins from wheat, rye and barley; precisely α/β-, γ-, ω1,2- and ω5-gliadins from wheat, ω-, γ-40k- and γ-75k secalins from rye as well as B-, C- and γ-hordeins from barley. The glutelins low-molecular-weight (LMW)-glutenin-subunits (GS) from wheat, high-molecular-weight (HMW)-GS from wheat, HMW-secalins from rye and D-hordeins from barley are not significantly detected by the R5 antibody.

Since the prolamins from rye and barley contain a higher copy number of the pentapeptide QQPFP [5], the R5 antibody has a higher reactivity against rye and barley compared with wheat, to which the RIDASCREEN® Gliadin R7001 is calibrated to (PWG gliadin). Wheat is by far the most commonly used gluten containing cereal in the world, so contamination of intended gluten-free products is very likely to occur with wheat. The main exception to this is oats, which is usually contaminated with barley, due to the geographic regions of cultivation, time of harvest and further processing. This leads to frequent overestimations of the gluten content in oat samples.

In order to address this issue, the AOAC has set up Standard Method Performance Requirements (SMPR®) for the quantitation of wheat, rye and barley gluten in oats [6]. Due to the overestimation of the R5 antibody, the RIDASCREEN® Gliadin R7001 does not fulfil these requirements and the development of a new ELISA with a more balanced quantitation of wheat, rye and barley was necessary.

Materials and methods

SMPR® 2017.021 reference materials [6] were obtained from Paul Wehling, General Mills, Minneapolis, USA. These materials consist of a set of seven samples: (1) one blank oat flour, (2) two oat flours spiked at levels of 10 mg/kg and 20 mg/kg wheat gluten; (3) two oat flours spiked at levels of 10 mg/kg and 20 mg/kg rye gluten, and (4) two oat flours spiked at levels of 10 mg/kg and 20 mg/kg barley gluten.

ELISA RIDASCREEN® Total Gluten (from R-Biopharm AG, Darmstadt, Germany) was used according to instructions for use. This ELISA contains the R5 antibody, one

monoclonal antibody raised against a known toxic sequence present on HMW-GS from wheat and HMW-secalins from rye and two monoclonal antibodies raised against a purified extract of LMW-GS proteins from wheat.

Purified gluten fractions LMW-GS, HMW-GS, rye prolamins and glutelins, barley prolamins and glutelins were obtained from Katharina Scherf, Leibnitz Institute for food system biology, Freising, Germany. The preparation of the material is described elsewhere [7]. The material was solubilised in Cocktail (patented) (from R-Biopharm AG, Darmstadt, Germany) and 80 % ethanol and diluted to suitable concentrations according to the instructions for use of the RIDASCREEN® Total Gluten.

Results and discussion

For the development of the new ELISA, it was decided to keep the R5 antibody for its high sensitivity to α/β-, γ- and ω1,2-gliadins from wheat, ω-, γ-40k- and γ-75k secalins from rye as well as B-, C- and γ-hordeins from barley. Additionally, the R5 recognises many peptides which were reported to be toxic for celiac disease patients [8, 9]. In order to reduce the overestimation of rye and barley gluten in oats, additional antibodies had to be combined with the R5 to counteract its high reactivity to rye and barley.

Since another limitation of the R5 antibody is that it does not react with other relevant gluten proteins (mainly glutelins), new antibodies against LMW-GS from wheat, HMW-GS from wheat and HMW-secalins from rye as well as D-hordeins from barley were raised. In the first attempt, reported toxic peptides from LMW-GS, HMW-GS and from D-hordeins were selected and used for monoclonal antibody generation. Different clones were obtained for all immunisations. However, only for HMW-GS, a suitable clone was identified. For LMW-GS, a second immunisation yielded two clones which in combination were suitable for LMW-GS detection. The clones for D-hordeins turned out to be not sensitive enough for usage.

Characterisation of all antibodies included their reactivity against different gluten fractions. For this, antibodies were used in homologue sandwich ELISAs (R5 as capture antibody on the microtiter plate and as detection antibody in the conjugate, LMW 1 on the plate and LMW 2 in the conjugate as well as HMW on the plate and in the conjugate, respectively).

As expected, the highest reactivity of the LMW antibodies was against the LMW-GS fraction from wheat (see Fig. 1A). Also the PWG gliadin showed an intermediate reactivity to the antibodies. This is also not surprising, since the PWG gliadin contains some wheat glutenins [10]. Additionally, the LMW-GS antibodies might also have a weak cross-reactivity against wheat prolamins.

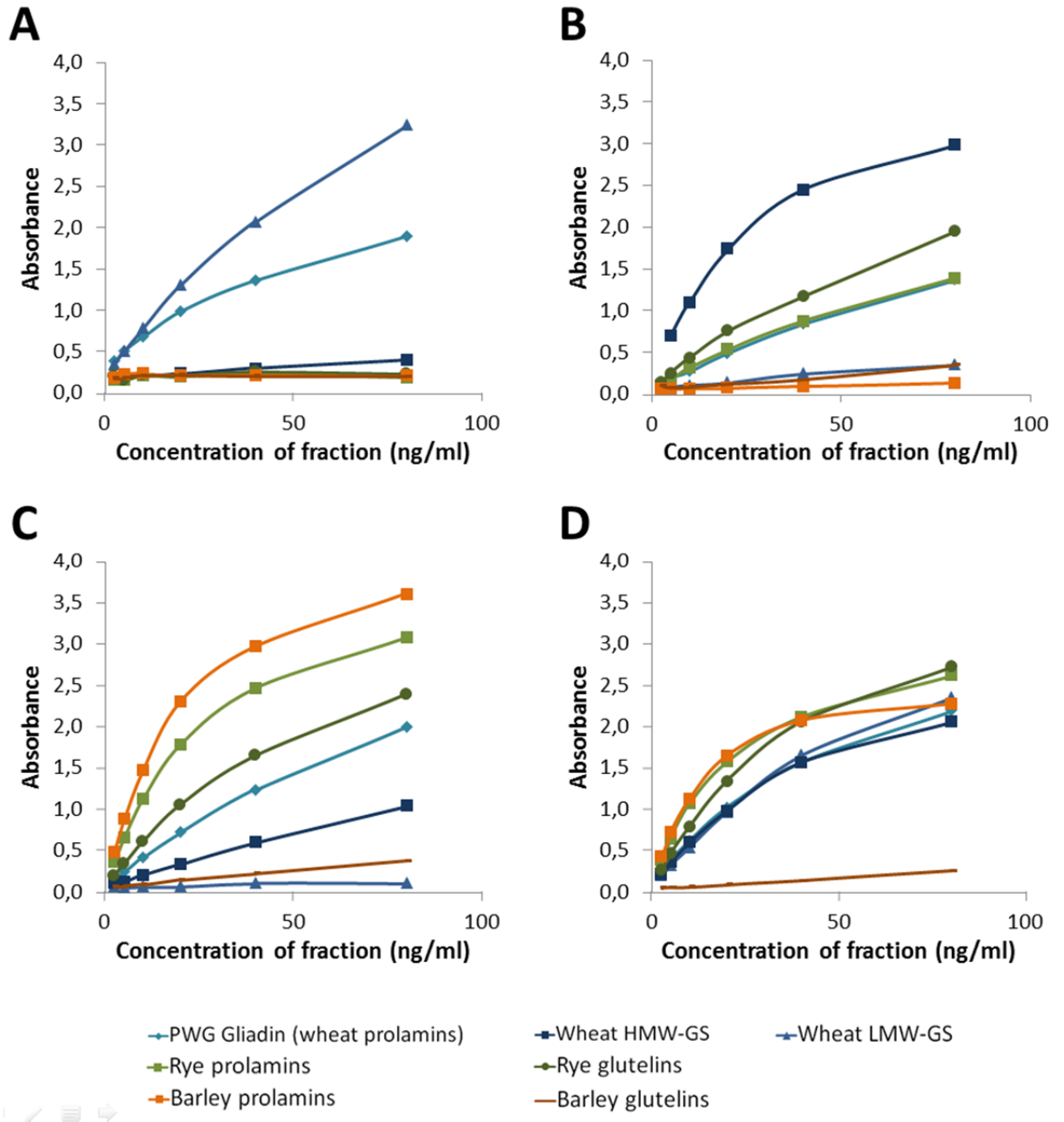

Figure 1. *Reactivity of the (A) LMW 1 and 2 antibodies (B) HMW antibody (C) R5 antibody and (D) the combination of all four antibodies in the RIDASCREEN® Total Gluten against different gluten fractions. Antibodies were used in separate sandwich ELISAs for (A), (B) and (C), and combined for (D). Fractions from wheat are depicted in blue colours (PWG in light blue, LMW-GS in medium blue and HMW-GS in dark blue), rye in green (rye prolamins in light green and rye glutelins in dark green) and barley in orange (barley prolamins in light orange, barley glutelins in dark orange).*

The HMW antibody showed the highest reactivity against the HMW-GS from wheat, followed by rye glutelins (see Fig. 1B). This was to be expected, as the immunisation peptide for the antibodies contained a sequence present on HMW-GS from wheat and HMW-secalins from rye, the latter being present in the rye glutelins fraction. The minor reactivity against PWG gliadin was also expected, since the PWG gliadin

contains some wheat glutenins [10]. The reactivity against the rye prolamins might also be due to some contamination of this fraction by HMW-secalins. Additionally, the HMW-GS antibody might also have a weak cross-reactivity against wheat and rye prolamins.

The reactivity of the R5 antibody was as expected and previously reported [7] with highest reactivity against prolamins from rye and barley (see fig 1C). Also rye glutelins showed a high signal, probably due to γ-40k- and γ-75k secalins, which contain the QQPFP sequence and are present in both prolamin and glutelin fraction [personal communication by Katharina Scherf]. In general, the exact separation of specific proteins into prolamins and glutelins in the course of the Osborne fractionation is not completely possible, as co-precipitation and co-solubilisation frequently occur [11, 12]. Intermediate reactivity was observed against PWG gliadin and low reactivity against HMW-GS from wheat. The latter was not expected, but might be due to some contamination by prolamin proteins.

In summary, each of the characterised antibodies recognised its target fraction with highest reactivity, and since the LMW antibodies and the HMW antibody showed highest reactivity against wheat fractions, these antibodies should be able to compensate the overestimation of the R5 to rye and barley. With these four antibodies, a combined sandwich ELISA was constructed with three antibodies combined in one well (R5, LMW 1 and HMW) and three antibodies combined in one conjugate (R5, LMW 2 and HMW). This combination was again tested for its reactivity against the different gluten fractions (see Fig. 1D).

The combination of the antibodies showed a very well balanced reactivity against the different gluten fractions. The only exception was the glutelins from barley (D-hordeins), since no antibody was expected and able to detect this fraction. However, the D-hordeins account for approx. 5 % of the barley gluten proteins only [12], so that only a very minor component cannot be detected. The combination of the four antibodies was further developed into a commercial product, the RIDASCREEN® Total Gluten with a 96 well microtiter plate coated with R5, LMW1 and HMW antibody in each well, ready to use standards containing 0/5/10/20/40/80 mg/kg gluten (standard material is an extract of four different wheat cultivars obtained from Katharina Scherf), ready-to-use conjugate with R5, LMW 2 and HMW antibodies conjugated to horse radish peroxidase, ready-to-use sample dilution buffer and a ten times concentrated washing buffer. The overall incubation time is 50 min. The result is given in mg/kg gluten as the sum result of prolamins and glutelins, thus the calculation from prolamin to total gluten as in the RIDASCREEN® Gliadin is not necessary any longer. This is a further advantage of this method, as the Codex factor of 2 for calculation from prolamins to total gluten proteins is inaccurate in most cases and leads to an overestimation [1, 13]. The extraction is performed using Cocktail (patented) in combination with 80 % ethanol. Final dilution factor for samples is 1000.

The new ELISA was tested for its reactivity against the SMPR® reference material: oat flours which were incurred with 10 and 20 mg/kg gluten from wheat, or rye, or barley,

respectively [6]. As figure 2 shows, the new ELISA has a very balanced detection of wheat, rye and barley. Three independent pilot lots of RIDASCREEN® Total Gluten were produced and tested for their lot to lot comparison. All tested samples showed very similar results in all three lots including the SMPR® reference material (data not shown). Further in-house validation is ongoing. Preliminary results indicate a Limit of Detection of approx. 2 mg/kg gluten and a Limit of Quantification of 5 mg/kg. More than 80 potentially cross-reacting substances were tested, none was found to show cross reactivity (data not shown).

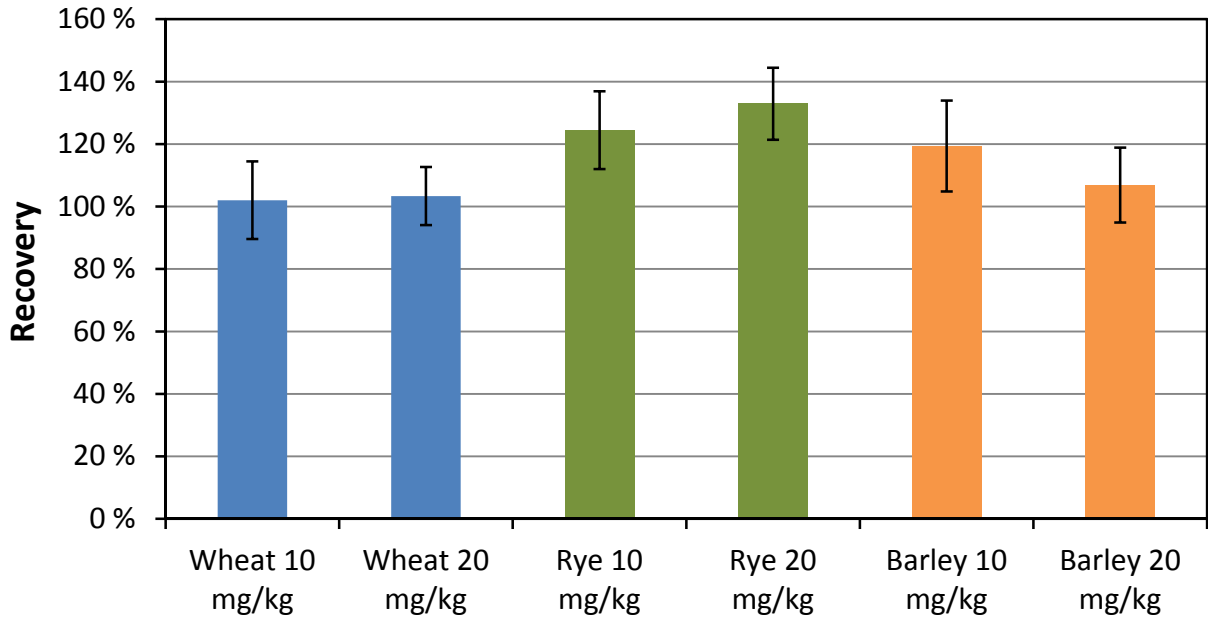

Figure 2. *Reactivity of AOAC SMPR® samples [6] in RIDASCREEN® Total Gluten. Each sample was extracted and analysed ten times. Wheat contaminated samples are depicted in blue colour, rye in green and barley in orange.*

An AOAC collaborative study with one of the pilot lots was performed in September 2018 with 19 laboratories worldwide using mainly oat samples. Preliminary results showed very good lab to lab comparison (data not shown). After completion of the in-house validation, statistical analysis of the collaborative study and production of the first lot in production scale, RIDASCREEN® Total Gluten will become commercially available in 2019.

Conclusions

The new ELISA RIDASCREEN® Total Gluten combines the well-established monoclonal R5 antibody with new antibodies against LMW-GS from wheat and against HMW-GS from wheat and HMW-secalins from rye. It is thus the first commercial ELISA targeting all major gluten fractions from wheat, rye and barley. The detection of all relevant fractions prevents inaccurate quantitation due to the

enrichment of a certain fraction during the processing of food, as it was reported for e.g. starches [13-15]. The result is given in mg/kg gluten and is the sum result of prolamins and glutelins; a calculation from prolamin content to total gluten content is not necessary. The new ELISA shows a very well balanced detection of wheat, rye and barley. The RIDASCREEN® Total Gluten is thus the ideal ELISA for analysis of oat samples.

In addition to in-house validation, an AOAC collaborative study has been performed in September 2018 using among other samples the SMPR® reference materials. Preliminary results support the in-house validation data, in particular the balanced detection of wheat, rye and barley.

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4.3 Development of a gluten reference material suitable for gluten analytical methods

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[5] *MoniQA Association, Güssing, Austria*

Introduction

Coeliac disease is an autoimmune hypersensitivity reaction causing mucosal damage and consequent absorption problems in the small intestine. The triggering components are the gluten proteins found in some cereals (wheat, rye, barley). The only effective treatment for the patients is a lifelong gluten-free diet [1]. Gluten-free (GF) products are available for them with a regulatory threshold of 20 mg/kg gluten content [2]. Various analytical methods can be applied to measure gluten contamination in GF products, but the most commonly used technique is the enzyme-linked immunosorbent assay (ELISA). However, accurate gluten determination is hindered by several factors. The protein content and composition of the cereals is not constant. The relative proportions of gluten protein fractions vary depending on genetic (species, varieties) and environmental factors (harvest year and agricultural practices). Additionally, their physical (e.g. solubility, structure) and (bio)chemical (e.g. reactivity, affinity) properties may change during food processing [3]. Commercially available ELISA tests provide partly different strategies for determining gluten concentration as they apply different extraction procedures, antibodies and target proteins and materials used for calibration. The problem is that we have limited information about the effect of the mentioned factors on the ELISA results and there is no certified reference material (RM) to compare the different measurement results and to validate the gluten analytical ELISA-based and alternative methods [4]. The production of a suitable RM raises a few questions: Are different species, a single cultivar or a mixture of several cultivars more suitable for this purpose? Is it necessary, and if yes, how can we take into consideration the effect of environmental factors (i.e. the stability) of protein composition? What form of protein sources - whole grain, flour or isolated protein - is suitable for RM formulation? Our research group deals with the issue of gluten RM

within the framework of international cooperation. Our aim is to investigate questions related to the production of RM and to choose and produce a gluten RM candidate.

Materials and methods

23 different wheat cultivars were collected from all over the world to investigate the effect of genetic variability. The applied workflow is demonstrated in Fig. 1. After full protein characterisation of the samples, different quality and quantity criteria were developed for selecting suitable varieties. These criteria related to the proper quantitative range of crude protein content, gliadin/glutenin ratio, α-gliadin/γ-gliadin ratio, gliadin recovery and the qualitative criteria related to the number of high-molecular-weight glutenin subunits and separation of γ-gliadins. Five cultivars were selected based on these criteria: Akteur from Germany, Carberry from Canada, Mv Magvas from Hungary, Yitpi from Australia and Yumay-34 from China [5]. The selected cultivars were collected from two harvest years (2014 and 2016) as well to include environmental variability as an additional factor of the study. The five different grains and their mixture of equal proportions were milled into white flours on laboratory scale at the Budapest University of Technology and Economics, Hungary (FQC 109 Micro-laboratory Mill, Metefém, Budapest, Hungary) and on pilot scale at AGES, Austria (Bühler MLU-202 Laboratory Flour Mill, Switzerland). The gluten and gliadin isolates were prepared from the flours on laboratory scale based on our developed protocol: gluten isolates were produced with the use of the Glutomatic System (Perten Instruments, Sweden), then gliadin was washed from dry gluten with 60 % (v/v) ethanol solution and freeze dried. Flours and protein isolates were characterised by their protein content obtained by the Dumas method (N x 5.7), and protein composition determined by SDS-PAGE, SE-HPLC [6] and RP-HPLC with modified Osborne fractionation [7]. The gluten protein concentrations were quantitated by RP-HPLC using PWG-gliadin as calibration reference [8]. The ELISA response of the samples was determined using two commercially available ELISA test kits: the AgraQuant Gluten G12 Assay (COKAL0200, Romer Labs, Tulln, Austria) and the RIDASCREEN Gliadin Assay (R7001, R-Biopharm, Darmstadt, Germany).

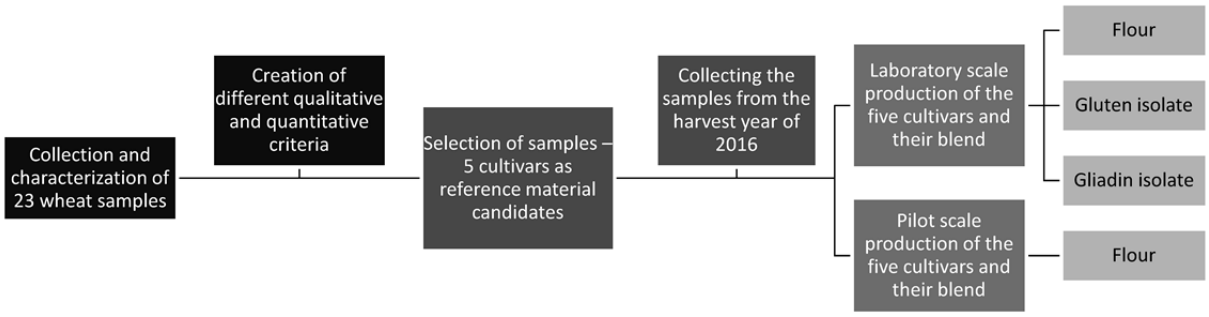

Figure 1. *Flowchart for the experimental work on the development of lab and pilot scale gluten reference material candidates*

The analytical results were statistically evaluated with the investigation of means, standard deviations, and factorial analysis of variance using Statistica 13 software (StatSoft Inc., Tulsa, USA). Correlation analysis was carried out by linear Pearson correlation at a confidence level of 0.95. The significance of differences was studied using t-tests.

Results and discussion

The flours of the selected five cultivars from two harvest years show great variability both in the crude protein contents and in the compositions according to separation techniques. This variability also appears in the gliadin concentrations measured by ELISA methods (Fig. 2). From the results, none of the varieties can be regarded as completely average. The protein content of the flours was typically higher in the year of 2016 than the year of 2014, so there were cultivars outside the quantitative parameters of the selection criteria. This shows that the effect of harvest year has a great influence on the amount of proteins and consequently the ELISA results, so it is difficult to choose one cultivar with stable protein content. Similar gliadin content was measured by the two ELISA methods, so using different methods has a smaller impact than the effect of genetics or harvest year (Fig. 2).

The blended flour represents well the average of the five selected samples resulting in a well-balanced genetic variability. Good homogeneity was obtained for it in each examined parameter, indicating that the applied lab scale homogenisation method is suitable for the production of blended flours. Additionally, the blended sample is best suited according to the selection criteria.

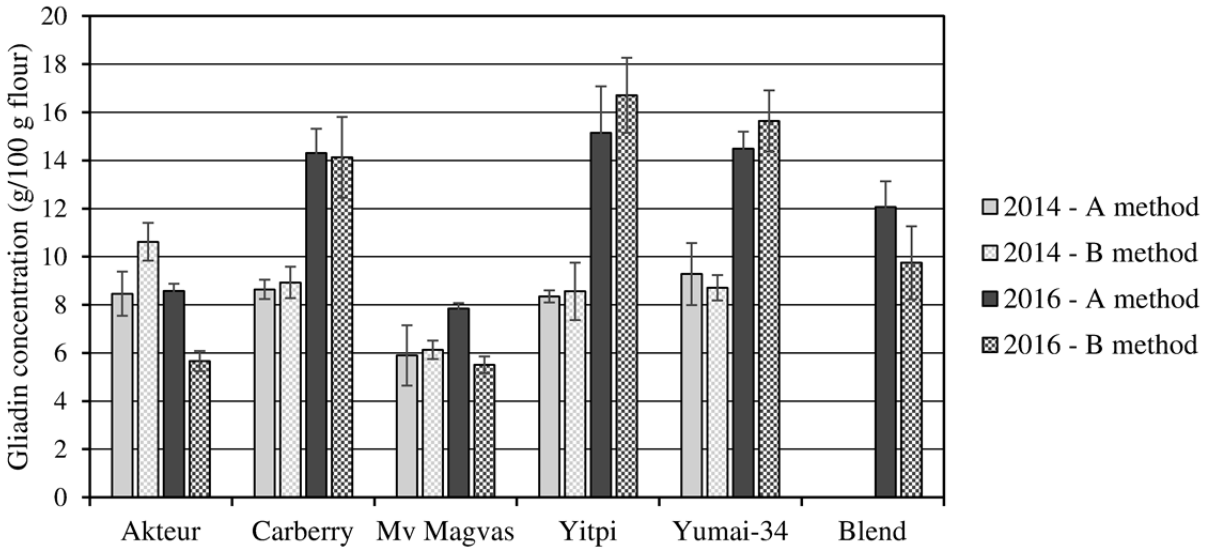

Figure 2. *Gliadin concentration of the five flour samples from two harvest years and their blend from one harvest year measured by two different ELISA kits (methods A and B)*

An essential criterion for a RM is the availability of an adequate amount of product. Therefore, upscaling of production was indispensable. According to our results, there was no significant difference between the flours produced at laboratory and pilot scale in the crude protein content and the ash content. Additionally, they had similar protein composition and gliadin/gluten content measured by ELISA. These results proved that we have succeeded in producing RM candidates in large scale (kg of material) which are almost equal to the lab scale flours, not cross-contaminated and homogeneous (data not shown).

As a second part of our work, gluten and gliadin isolates were prepared in laboratory scale and compared to the flours of origin. The protein profiles of the isolates were similar to the flours and there was no significant difference between the ELISA recovery values of flours and their gluten isolates. Flours and gliadin isolates differed significantly in just a few cases (Fig. 3). Therefore, loss of analytical information should not be expected as a result of isolation.

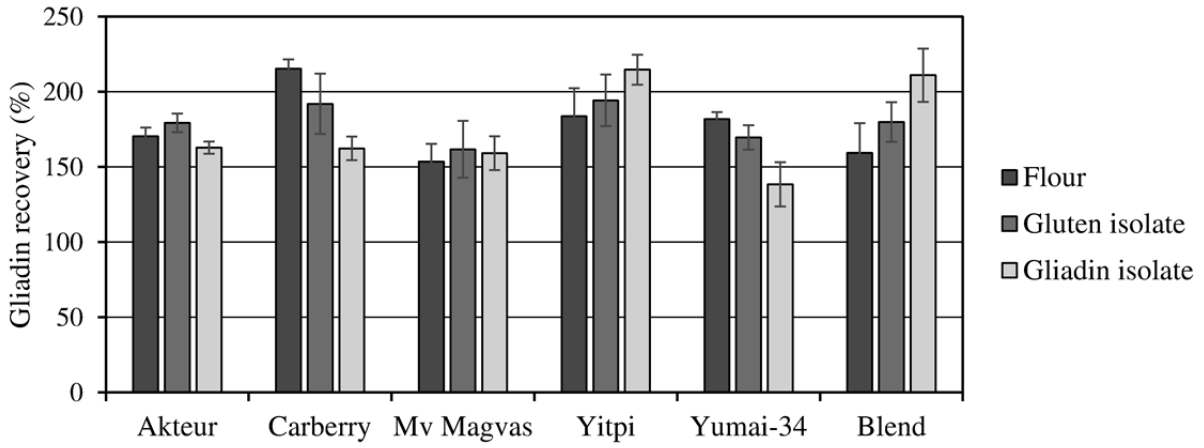

Figure 3. *Gliadin recovery values of the six flours and their gluten and gliadin isolates with ELISA kit A (calculation based on gliadin content measured by RP-HPLC)*

Conclusions

During our research we examined questions related to the production of a proper RM for gluten analysis. After a preliminary selection process, five wheat cultivars were chosen. Lab scale and pilot scale flours and lab scale gluten and gliadin isolates were characterised by determination of protein composition and epitopes via ELISA methods. Our results confirmed that the mixture of varieties can compensate the genetic and environmental variabilities of individual varieties. Our upscaled production procedure seemed to be successful, showing that we are able to produce flour mixture-based reference candidate materials in pilot-scale.

In our experiments, flours and isolates gave similar results in protein composition and ELISA response as well. Consequently, the decision about the application of flour over

isolates in a RM should be based on other considerations (such as stability, solubility, analytical application, etc.). Studies on these issues are still ongoing as a contribution to solve this long-standing analytical problem of gluten RM.

Outlook

An international collaborative study will be organised to validate the selected flour mixture and it is expected to be available for users in 2019. The last step of our research in the production of wheat-based reference material is the production of a processed incurred reference material modelling real food matrices and examining the effects of food processing on the ELISA results.

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4.4 Comparative reactivity of avenins from different pure oat varieties to gluten R5 and G12 ELISA immunomethods

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Introduction

Gluten derived from wheat, barley and rye trigger autoimmune gut mucosal damage in coeliac disease (CD). However, the role of gluten from oat (avenins) has been controversial for many years, as evidenced by the lack of consensus in the legislation of gluten-free products. The difference in type of oats used, their purity and study designs have not allowed a clear answer as to whether oats are safe or not for all CD patients [1]. To simplify the problem, it has been broadly accepted that pure oats are safe for most CD patients, and contamination with other cereals is the main problem to face [2,3]. However, it has been shown in different studies that some varieties of oat may contain certain levels of peptides that can trigger immunoresponse in CD patients or cross-reactivity in gluten immunomethods used for food labelling [1,4-7]. One of the common assumptions by many stakeholders is that oats are naturally gluten-free and only when oats are contaminated with other immunotoxic cereals as wheat or barley, reactivity in gluten ELISA tests could be observed [2,3]. However, some pure oats cultivars have shown significant cross-reactivity with G12, A1, Skerritt and R5 antibodies [1,4-6]. Some coeliac T-cell activating sequences from oat have been identified [4,5,7] and some oat varieties have elicited early inflammatory events typical of CD [3-5,7,8]. There are some clinical studies where gluten challenges were made with oats that described adverse effects in some volunteers of the clinical studies [2]. In this work, we showed that the cross-reactivity of some pure oat cultivars with R5 or G12 antibodies significatively correlated with the immunogenicity estimated by c0eliac T cell activation.

Materials and methods

Oats (*Avena sativa* L.) from cultivars designated OE717, OA729, OM719, OC723, OH727, and OL715 (obtained from Spanish and Australian commercial sources) were used in this work. The purity of the oat cultivars were checked by visual inspection and PCR amplification with primers described elsewhere [1]. These cultivars were

chosen based on their previously reported CD immunoactivity [1]. The immunoassays were performed from the guidelines of the manufacturer (GlutenTox ELISA Sandwich A1-G12 Hygiena Diagnostica, Seville, Spain; Ingenzim ELISA R5, Madrid, Spain).

Peripheral blood mononuclear cells (PBMCs) and cell cultures from 10 patients with active CD on a gluten-containing diet were isolated from 6 ml of heparinised blood by Histopaque gradient centrifugation and cultured at a density of $1X10^6$ cells/ml in RPMI-1640 culture medium. After 48 h, PBMCs were incubated with avenin, gliadin and oryzenin peptides (50 μg/ml).

Cell proliferation analysis and interferon gamma (IFN-γ) production were made according to Comino et al. [1]. IFN-γ production by T cells with prolamin digests from three different oat varieties. T lymphocytes were stimulated with digested prolamins after treatment with tissue transglutaminase. IFN-γ production was evaluated by ELISA after 48 h of incubation. The results are shown as the means of duplicate wells and expressed as pg/ml. Gliadin and oryzenin were used as the positive and negative control, respectively.

Results and discussion

We compared the reactivity of oat cultivars to the R5 and G12 antibodies (moAbs). These seven oat cultivars, together with gliadin and oryzenin controls (Tab. 1) were analysed for gluten content using sandwich R5 Ingezim and sandwich GlutenTox A1-G12.

Table 1. *Immunoreactive gluten content in different pure oat cultivars and control samples estimated by ELISA R5 and G12 moAbs and by T cell immunogenicity*

	Gluten content estimated with gliadin standard (ppm)		
Sample[a]	**ELISA R5/R5**	**ELISA A1/G12**	**T-cell reactivity**[b]
Oat OM719	583	503	++
Oat OA729	901	874	NT[c]
Oat OH727	407	86	+
Oat OL715	46	53	NT[c]
Oat OC723	<LQ[d]	<LQ[d]	-/+
Oat OR721	<LQ[d]	<LQ[d]	NT[c]
Gliadin	>100,000	>100,000	+++
Oryzenin	<LQ[d]	<LQ[d]	-

[a] The reference of the cultivars is internal to protect undisclosed information about the cultivars.

[b] The relative immunogenicity was estimated by activation of T cell division and interferon γ expression levels in PMBCs isolated from coeliac patients. The (-) symbol was the least reactive and the more (+) symbols, the more level of immunoreactivity of the sample.

[c] NT, Not tested

[d] LQ, limit of quantification

Cultivars named OM719, OA729, OH727 and OL715 showed detectable levels of gluten for both moAbs. However, in the case of OM719 was about 13- and 10-fold more reactive than the OL715 with the moAbs R5 and G12, respectively. The cultivars OC723 and OR721 presented levels of gluten below 10 ppm with R5 and G12 moAbs (Tab. 1), similar to those of the oryzenin negative control. In any case, the most reactive oat cultivars (OA729) contained hundreds to thousand-fold less reactivity than gliadin for the R5 or G12 moAbs.

The immunogenicity with coeliac volunteers T cells was analysed in gliadin as positive control, oryzenin as negative control, and three representative oat cultivars: the one with the highest G12/R5 reactivity, OM719, the one with differential reactivity between R5 and G12, OH727, and one of the lowest reactivity to both G12 and R5, OC723. Gliadin and prolamins from OM719 were very immunogenic, with the highest values of IFN-γ release (9.4 ± 0.76 and 7.9 ± 0.57 pg/ml, respectively), whereas the exposure to OH727 induced a lower mean value of IFN-γ (4.8 ± 0.95 pg/ml). Finally, OC723 and oryzenin were the least immunogenic (3.4 ± 1.09 and 2.3 ± 0.89 pg/ml, respectively).

Conclusions

The immunogenicity of the analysed pure oat cultivars estimated by T-cell activation correlated with the cross-reactivity to the main used monoclonal antibodies used to detect gluten in food. Assuming the caution of the low number of analysed immunogenic oats, the G12 reactivity correlated slightly more to the degree of immunogenicity of the oat varieties because it could distinguish levels of differential immunogenicity in the 3 out of 3 of the studied cultivars; 2 out of 3 in the case of R5. The absence of reactivity to G12 antibodies may then indicate low risk of potential immunotoxicity due to either the absence of toxic cereal contaminants or very low content of immunogenic oat peptides.

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4.5 Targeted LC-MS/MS reveals similar contents of α-amylase/trypsin-inhibitors in all wheat species except einkorn

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Introduction

Among wheat hypersensitivities, non-celiac gluten sensitivity (NCGS) is least well-defined, because the causative factors have not been unambiguously identified, specific biomarkers are missing and the diagnosis relies on exclusion of coeliac disease (CD), wheat allergies, other food intolerances and irritable bowel syndrome [1]. The estimated prevalence ranges from 0.6 – 6 % of the population. The symptoms occur several hours until a few days after consumption of wheat products and include intestinal as well as extraintestinal (e.g., lack of wellbeing, tiredness, headache, anxiety, foggy mind and joint/muscle pain) complaints. Upon treatment with a gluten-free diet (GFD), the symptoms disappear [2]. In contrast to CD, NCGS patients have a normal small intestinal mucosa, no autoantibodies and there does not appear to be a relation to the expression of HLA-DQ2 or -DQ8, but they had increased numbers of intraepithelial lymphocytes (IELs), levels of toll-like receptor (TLR) 2 and TLR4 and reduced numbers of regulatory T cells, all markers of the innate immune response [3].

The pathomechanism of NCGS remains poorly understood. Potentially harmful wheat components include gluten proteins, α-amylase/trypsin-inhibitors (ATIs), wheat germ agglutinins and fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs). ATIs from gluten-containing cereals were found to induce the innate immune response through activation of the TLR4-MD2-CD14 complex on monocytes, macrophages and dendritic cells resulting in release of the pro-inflammatory cytokines and chemokines IL-8, TNF-α and C-C motif chemokine ligand 2 (CCL2). ATIs were also identified as adjuvants of pre-existing inflammatory adaptive immune responses [4,5]. These ATI-induced innate immune responses are dose-dependent, suggesting that a reduction of nutritional ATI intake may be sufficient to prevent inflammation. Until the pathomechanism of NCGS is elucidated in more detail, NCGS patients are advised to follow a GFD or a gluten-reduced diet.

ATIs make up about 2 - 4 % of wheat proteins and have been identified as major causative factors of baker's asthma. In the plant, they serve as defence proteins against pests. Thirteen different ATI types (UniProtKB: 0.19, 0.28, 0.53, CM1, CM2, CM3,

CM16, CM17, CMX1/3, CMX2, wheat subtilisin inhibitor, Bowman-Birk type trypsin inhibitor and chymotrypsin inhibitor WCI) have evidence at protein level in common wheat (*Triticum aestivum* L., hexaploid) [6]. Bioactivity assays with TLR4-expressing monocytes showed that ATI extracts from spelt (*T. spelta* L., hexaploid), emmer (*T. dicoccum* L., tetraploid) and einkorn (*T. monococcum* L., diploid) only had 30 – 70 % of the activity of common wheat and it has been reported that einkorn had either very low amounts of ATIs or even none [7,8].

All of the above led to the hypothesis that spelt, emmer and einkorn may be better tolerated by NCGS patients, because of lower ATI contents compared to common and durum wheat (*T. durum* L., tetraploid). To test this hypothesis, a targeted LC-MS/MS method using stable isotope labelled peptides as internal standards (stable isotope dilution assay, SIDA) was developed to quantitate the predominant ATIs 0.19, 0.28, 0.53, CM2, CM3 and CM16 in eight well-characterised common wheat, durum wheat, spelt, emmer and einkorn cultivars, respectively.

Materials and methods

Grain samples

Eight cultivars each of common wheat, spelt, durum wheat, emmer and einkorn were cultivated and harvested in 2013 by Friedrich Longin (State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany) at Seligenstadt, Germany [9]. The grains were milled into wholemeal flours using a cross-beater mill (Perten Instruments, Hamburg, Germany) and analysed for water, ash, crude protein and albumin/globulin, gliadin and glutenin contents [10].

Sample preparation for targeted LC-MS/MS and SIDA

Flour (50 mg) was extracted twice for 30 min at 22 °C with ammonium bicarbonate (Abic) solution (0.5 mL, 50 mmol/L, pH 7.8). The suspensions were centrifuged for 25 min at 3750 × *g*, the supernatants combined and dried. The residue was dissolved in Tris-HCl (320 µL, 0.5 mol/L, pH 8.5) and 1-propanol (320 µL). The five heavy labelled peptides as internal standards (IS) were added (Tab. 1), followed by reduction with tris(2-carboxyethyl)phosphine (TCEP), alkylation with chloroacetamide (CAA) and lyophilisation. Tryptic hydrolysis (enzyme-to-substrate ratio 1:50) was performed for 24 h at 37 °C in the dark. Having stopped the reaction with trifluoroacetic acid, the solution was evaporated to dryness. The residue was dissolved in 0.1 % formic acid (FA), filtered (0.45 µm) and used for targeted LC-MS/MS [11].

Targeted LC-MS/MS and SIDA

An UltiMate 3000 HPLC system (Dionex, Idstein, Germany) coupled to a triple-stage quadrupole mass spectrometer (TSQ Vantage, ThermoFisher Scientific, Bremen, Germany) was used.

Table 1. *Amino acid sequences of α-amylase/trypsin-inhibitor (ATI) marker peptides (P) and internal standards (IS), and single reaction monitoring (SRM) parameters (precursor ions and product ions).*

No.	Amino acid sequence	ATI-type	Precursor ions	Product ions
P1	LQCNGSQVPEAVLR	0.19 + 0.53	786.3 (2^+)	684.3 (y6) 783.5 (y7) 1330.8 (y12)
IS1	LQCNGSQV*PEA*VLR	0.19 + 0.53	792.3 (2^+)	696.4 (y6) 795.6 (y7) 1342.7 (y12)
P2	LQCVGSQVPEAVLR	0.28	778.7 (2^+)	684.0 (y6) 1055.7 (y10) 1315.3 (y12)
IS2	LQCVGSQV*PEA*VLR	0.28	784.7 (2^+)	696.4 (y6) 1067.8 (y10) 1327.5 (y12)
P3	EYVAQQTCGVGIVGSPVSTEPGNTPR	CM2	902.2 (3^+)	641.5 (y6) 958.6 (y9) 1154.4 (y11)
IS3	EYVAQQTCGVGIVGSPVSTE*P*GNT*PR	CM2	907.2 (3^+)	656.6 (y6) 974.6 (y9) 1170.9 (y11)
P3c	qYVAQQTCGVGIVGSPVSTEPGNTPR	CM2	896.0 (3^+)	641.5 (y6) 958.6 (y9) 1154.4 (y11)
IS3c	qYVAQQTCGVGIVGSPVSTE*P*GNT*PR	CM2	901.0 (3^+)	656.6 (y6) 974.6 (y9) 1170.9 (y11)
P4	SGNVGESGLIDLPGCPR	CM3	864.8 (2^+)	585.9 (y5) 699.3 (y6) 1185.7 (y11)
IS4	SGNVGESGLIDL*PGC*PR	CM3	870.8 (2^+)	598.3 (y5) 711.4 (y6) 1197.4 (y11)
P5	QQCCGELANIPQQCR	CM16	931.8 (2^+)	688.6 (y5) 986.5 (y8) 1099.4 (y9)
IS5	QQCCGE*LANI*PQQCR	CM16	938.3 (2^+)	694.3 (y5) 992.5 (y8) 1112.8 (y9)
P5c	qQQCCGELANIPQQCR	CM16	922.9 (2^+)	688.2 (y5) 986.5 (y8) 1099.4 (y9)
IS5c	qQCCGE*LANI*PQQCR	CM16	929.4 (2^+)	694.2 (y5) 992.5 (y8) 1112.8 (y9)

* P, proline ($^{13}C_5$, ^{15}N); *V, valine ($^{13}C_5$, ^{15}N); *G, glycine ($^{13}C_2$, ^{15}N); *L, leucine ($^{13}C_6$, ^{15}N); post-translational modifications: q, pyroglutamyl; C, S-carboxamidomethylcysteine

Peptides were separated on an Aqua®-C_{18} column (50 × 2 mm, 5 µm, 12.5 nm, Phenomenex, Aschaffenburg, Germany) with the following LC conditions: solvent A, FA (0.1 %, v/v) in water, solvent B, FA (0.1 %, v/v) in acetonitrile; gradient, 0 - 5 min

10 % B, 5 - 20 min 10 - 90 % B, 20 - 23 min 90 % B, 23 - 25 min 90 - 10 % B, 25 - 40 min 10 % B; flow rate, 0.2 mL/min; injection volume, 10 µL; column temperature, 22 °C. The ion source was operated in the ESI positive mode with optimised source parameters. Selected reaction monitoring (SRM) was used to analyse the transitions from precursor to product ions (Tab. 1). Response lines were plotted by linear regression of the peak area ratios A(P1-P5)/A(IS1-IS5) against the molar ratios n(P1-P5)/n(IS1-IS5). Precision, limits of detection (LOD) and quantitation (LOQ) and recovery of the method were determined as described by Geisslitz et al. [11].

Results and discussion

Method development for targeted LC-MS/MS and SIDA

First, untargeted LC-MS/MS analysis was used to identify the final five marker peptides for ATIs 0.19+0.53, 0.28, CM2, CM3 and CM16 (Tab. 1). The peptides were unique for each ATI type, except for P1 that occurred in 0.19 and 0.53. Suitable precursor ions were identified by recording full-scan mass spectra of P1-P5 and IS1-IS5 and the selected precursor ions were fragmented to define the three most abundant transitions for selected reaction monitoring (SRM). The slopes of the response lines with n(P)/n(IS) between 9.1 and 0.1 were between 0.7 and 1.2 and the intercepts were close to 0.0, as expected for SIDA. Overall, the method showed good performance characteristics in terms of repeatability (coefficient of variation, 2 - 5 %), intermediate precision (coefficient of variation, 3 - 7 %), LODs (0.1 - 1.6 µg/g), LOQs (0.3 - 4.7 µg/g), recovery assessed by spiking peptides into an analyte-free cassava starch matrix (76 - 121 %) and recovery assessed by diluting common wheat flour with cassava starch (92 - 102 %).

Application of the SIDA to the collection of flour samples (all ranges given as lowest and highest values of the eight cultivars per wheat species) showed that the contents of 0.19+0.53 were significantly higher in common wheat (1.47 - 1.89 mg/g) and spelt (1.51 - 2.08 mg/g) compared to durum wheat (0.84 - 1.15 mg/g) and emmer (0.90 - 1.25 mg/g). The contents of 0.28 were similar in common wheat (0.31 - 0.40 mg/g), spelt (0.30 - 0.55 mg/g) and emmer (0.24 - 0.33 mg/g), but high variability was observed in durum wheat, with two cultivars containing 0.21 mg/g (cv. LUN, Lunadur) and 0.26 mg/g (cv. WIN, Wintergold) and the other six cultivars containing amounts near the LOD of 6.6 µg/g. Emmer (0.37 - 0.58 mg/g) had the highest contents of CM2, followed by durum wheat (0.29 - 0.54 mg/g), common wheat (0.17 - 0.21 mg/g) and spelt (0.20 - 0.30 mg/g). The contents of CM3 were significantly higher in spelt (1.07 - 1.59 mg/g), durum wheat (0.95 - 1.88 mg/g) and emmer (1.32 - 1.98 mg/g) compared to common wheat (0.77 - 0.98 mg/g). Durum wheat (1.15 - 2.07 mg/g) and emmer (1.38 - 2.10 mg/g) had higher contents of CM16 than common wheat (0.62 - 0.81 mg/g) and spelt (0.76 - 1.21 mg/g). This resulted in overall ATI contents (sum of the five types) of 3.4 - 4.1 mg/g in common wheat similar to durum wheat (3.2 - 5.5 mg/g), but lower than in spelt (4.1 - 5.7 mg/g) and emmer (4.4 - 6.3 mg/g). The ATI contents of einkorn were between the LOD

(40.5 µg/g) and 0.3 mg/g, thus confirming the absence or presence of very low amounts of ATIs in einkorn.

The initial hypothesis was thus confirmed for einkorn, but refuted for spelt and emmer, because the ATI contents were significantly higher than those of common wheat and durum wheat. The contents of ATIs were not correlated to those of albumins/globulins or crude protein. Principal component analysis (PCA) based on contents of each individual ATI, total ATI, albumins/globulins and crude protein showed that einkorn cultivars distinctly clustered together opposite of the vector belonging to total ATI contents (Fig. 1). Common wheat and spelt cultivars were mostly located in the lower right rectangle, whereas emmer and durum wheat cultivars were located in the upper right rectangle, with only two spelt cultivars in between. The ratio of CM-ATIs to the sum of 0.19, 0.28 and 0.53 was about 1:1 in hexaploid wheat species, but around 3:1 in tetraploid wheat species, so that the distribution of ATIs seemed to be suitable to differentiate hexaploid from tetraploid wheat species.

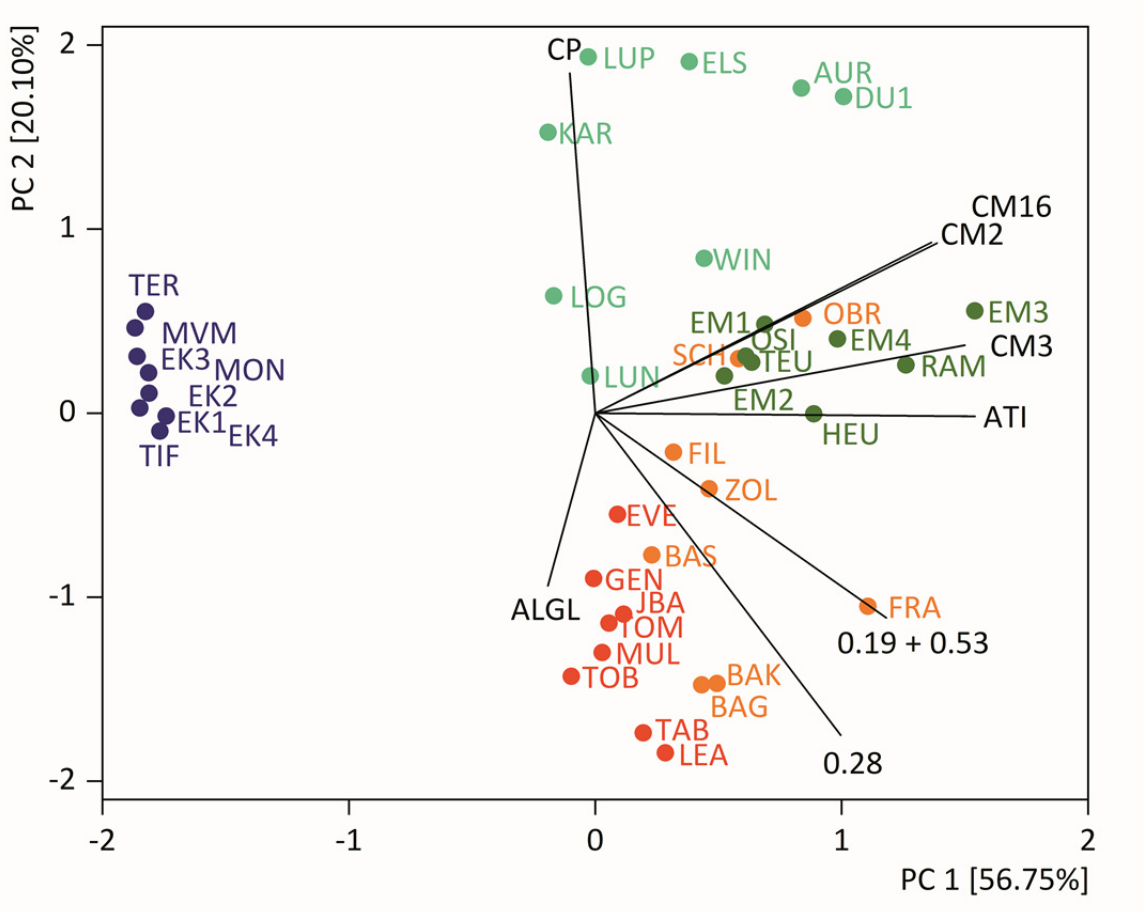

Figure 1. *Principal component analysis biplot of data for contents of single ATIs 0.19 + 0.53, 0.28, CM2, CM3 and CM16, total ATIs (ATI), albumins/globulins (ALGL) and crude protein (CP). Flours of eight common wheat (red), spelt (orange), durum wheat (light green), emmer (dark green) and einkorn (dark purple) cultivars each grown at the same location were analysed. PC, principal component. Figure modified from [11].*

Conclusions

The quantitative analysis of ATIs in five wheat species and eight cultivars each grown under the same environmental conditions by targeted LC-MS/MS revealed that einkorn contains very low amounts of the ATIs 0.19+0.53, 0.28, CM2, CM3 and CM16. Compared to common wheat, spelt and emmer had higher contents of ATIs, with durum wheat in between. Based on these results, it may be possible that einkorn products are better tolerable for NCGS patients. Further in-depth investigations using well-defined grain samples are necessary to substantiate this dataset and relate ATI contents to in vivo bioactivity using markers of inflammation.

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4.6 Pathogenesis of coeliac disease: Identification of isopeptides by LC-MS/MS

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Introduction

Coeliac disease (CD) is one of the most frequent food hypersensitivities affecting approximately 1 % of the population worldwide [1]. This disease can be characterised by three features: a) triggered by the ingestion of gluten, b) presence of the genetic factor (HLA-DQ2 or DQ8), and c) generation of autoantibodies against tissue transglutaminase (TG2) [2]. The gluten proteins are not sufficiently digested by the human gastrointestinal enzymes, so long gluten peptides pass through the epithelial layer and first trigger the innate immune response. Intraepithelial lymphocytes activate defence mechanisms, which initiate apoptosis and increase epithelial permeability. Secondly, TG2 modifies the gluten peptides by deamidation and transamidation. The modified peptides stimulate gluten-specific T-lymphocytes, which finally lead to the damage of the villi of the small intestine. Furthermore, antibodies are formed against gluten peptides, TG2 and gluten peptide-TG2-complexes [2-4].

The two reactions of TG2 play a key role in the pathogenesis of CD. On the one hand, the deamidation of specific glutamine residues to glutamic acid increases the immune response. On the other hand, the transamidation and formation of gluten peptide-TG2-complexes leads to the formation of antibodies against them. TG2 is a Ca^{2+}-dependent protein-glutamine γ-glutamyltransferase (EC 2.3.2.13), which catalyses the formation of inter- and intramolecular N^{ε}(γ-glutamyl)lysine bonds. The reactions take place in the active site of the enzyme with the three amino acids cysteine-277, histidine-335, and aspartic acid-358.

The aim of this study was to develop an analysis method by LC-MS/MS and to identify isopeptides and their binding sites in the TG2-gluten peptide-complexes in a model system.

Materials and methods

Model system of TG2 and the CD-active PepQ

The transamidation reaction of TG2 with the model peptide PFPQPQLPY-NH_2 (PepQ) was performed in 0.1 mol/l TRIS/HCl buffer (pH 7.4, 20 mmol/l $CaCl_2$) at a molar ratio of 1:150 at 37 °C for 120 min. To inactivate TG2, all samples were heated at

95 °C for 10 min. The samples containing the TG2-PepQ-complexes and the negative controls were hydrolysed with trypsin and purified by solid phase extraction (SPE) using 50 mg Sep-Pak tC18 cc cartridges (Waters, Eschborn, Germany). The isopeptides and peptides were eluted with acetonitrile/water/formic acid (FA) (40:60:0.1; 1 ml), dried and reconstituted in FA (0.1 %, v/v). For MS, the peptide concentrations of the reconstituted samples were estimated with a NanoDrop Micro-UV/VIS spectrophotometer (NanoDrop One, Thermo Scientific, Madison, USA) at 280 nm. The samples and negative controls were diluted in the 96 well plates to a concentration of 200 ng/µL with acetonitrile/water/FA (2:98:0.1, v:v:v).

LC-MS/MS analysis was performed on an Ultimate 3000 nanoHPLC system (Dionex, Idstein, Germany) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany). The nanoscale LC system is composed of a trap column (75 µm x 2 cm, self-packed with Reprosil-Pur C18 ODS-3 5 µm resin, Dr. Maisch, Ammerbuch, Germany) and an analytical column (75 µm x 40 cm, self-packed with Reprosil-Gold, C18, 3 µm resin, Dr. Maisch). The injection volume was 5 µL. The peptides were delivered to the trap column using solvent A0 (0.1 % FA in water) at a flow rate of 5 µL/min and were then separated on the analytical column using a 60 min linear gradient from 4 % to 32 % solvent B at a flow rate of 300 nL/min (solvent A1, 5 % DMSO, 0.1 % FA in water; solvent B, 5 % DMSO, 0.1 % FA in acetonitrile) [5]. The mass spectrometer was operated in data dependent acquisition mode, automatically switching between MS and MS^2 spectra. The mass-to-charge (m/z) range of the acquisition of the MS^1 spectra was 360 - 1300 m/z at an Orbitrap full MS scan (60,000 resolution, 3e6 automatic gain control (AGC) target value, 50 ms maximum injection time). In the MS^2, peptide precursors were selected for fragmentation by higher energy collision-induced dissociation (HCD; isolation width of 1.7 Th, maximum injection time of 25 ms, AGC value of 1e5). Analysis was performed using 25 % normalised collision energy (NCE) at a resolution of 15,000.

Data analysis of isopeptides

Data analysis was carried out with the Thermo Xcalibur .raw files in the MaxQuant software (version 1.6.0.1) to search individual LC-MS/MS runs against the model-system-specific database of α/β-gliadin. The TG2-isopeptide sites were configured as modifications in MaxQuant by calculating the empirical formulas of the lysine-containing tryptic TG2-peptides (UniProt accession no. P21980). To use these peptides as modifications (TG2-modifications) and to simulate an isopeptide bond formation, a formal subtraction of NH_3 was necessary. A theoretical protease for PepQ and the model-specific database had to be configured with the following cleavage specificity: QP, QY. For the individual search runs all parameters were used as default, except the following settings: digestion mode: specific; maximum missed cleavage sites: 2; variable modifications: every TG2-modification in one single search run; Fasta file: UniProt accession no. P18573; fixed modifications: amidated C-term; minimum score for modified peptides: 40; main search peptide tolerance: 4.5 ppm; mass tolerance for fragment ions: 0.5 Da. The MaxQuant Viewer was used to confirm the identification

of the isopeptides and the identification of the binding sites within the isopeptide by assigning the b- and y-fragments of both sites to the same MS/MS spectrum.

Results and discussion

Identification of isopeptides

To identify the isopeptides in the model system, TG2 was incubated with PepQ. The mix was then hydrolysed with trypsin, purified and subsequently measured by nanoLC-MS/MS. The raw data were searched for PepQ with the TG2-modifications and an identification score of > 100 was applied. With our data analysis strategy, we were able to identify 22 different isopeptides. Fig. 1 presents the chromatogram of 21 isopeptides with the binding lysines indicated. For the isopeptide with the modification "K" it was not possible to illustrate the chromatogram for technical reasons.

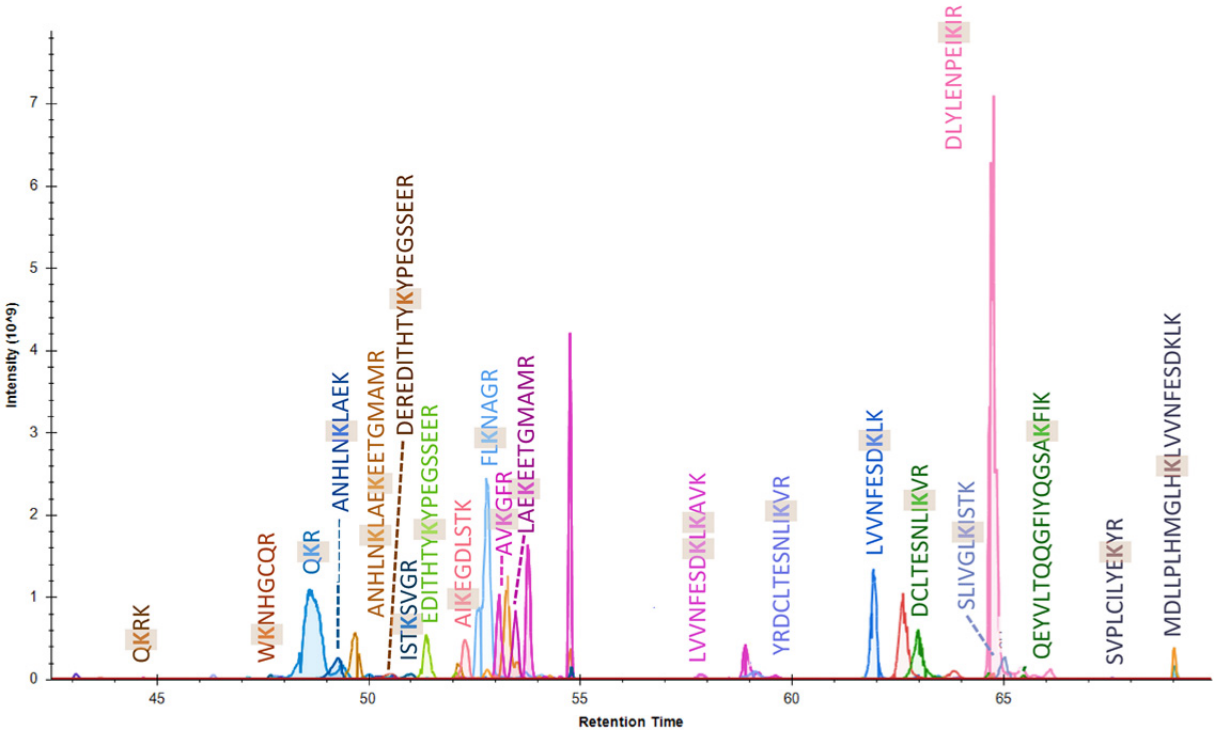

Figure 1. *Chromatogram of 21 identified isopeptides. The TG2-site is shown, respectively.*

Within these 22 isopeptides, we were able to identify isopeptides with 6 known [6] and 11 new lysine binding sites of TG2. The 3D-model of the TG2 with its different domains and the positions of the binding site is shown in Fig.2.

The three amino acids of the active site are marked in blue; they are located in the core region. The lysines, which are involved in an isopeptide bond, are marked in orange and are located more on exposed positions.

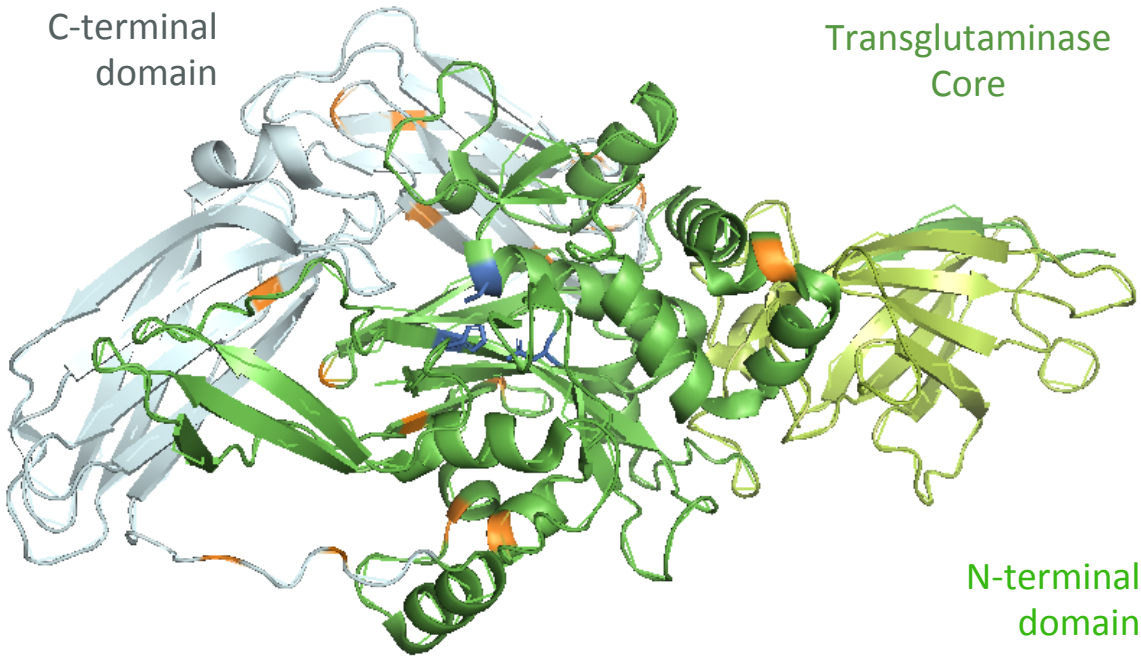

Figure 2. *3D-model of TG2 with the C-terminal domain, the core region and the N-terminal domain. The amino acids of the active site are marked in blue, the identified lysines are marked in orange.*

The single isopeptides were identified with the data analysis strategy developed using MaxQuant. The raw-files were searched from both sites, first against the TG2-FASTA-file with PepQ-modifications and secondly against the PepQ-FASTA-file with TG2-modifications. With the software tool MaxQuant Viewer it was possible to assign the identified b- and y-fragments to the mass-spectrum.

For the annotation of both sides the same scan number was taken. In Fig. 3 two related mass spectra are shown. On the upper left side the b- and y-fragments of the modified PepQ are annotated to the spectrum and in the lower right corner the spectrum with the fragments of the modified TG2-peptide LEAKEETGMAMR is displayed.

Conclusions

A software-supported and database-assisted proteomics search strategy with MaxQuant and the search engine Andromeda to identify isopeptides between TG2 and gluten-derived model peptides was established. Until now, six known [6] and 11 new lysines of TG2 were identified as isopeptide binding sites.

Further work will focus on the improvement and advancement of the method for the identification of isopeptides in gluten protein samples to identify the binding sites in the gluten proteins of wheat, rye and barley.

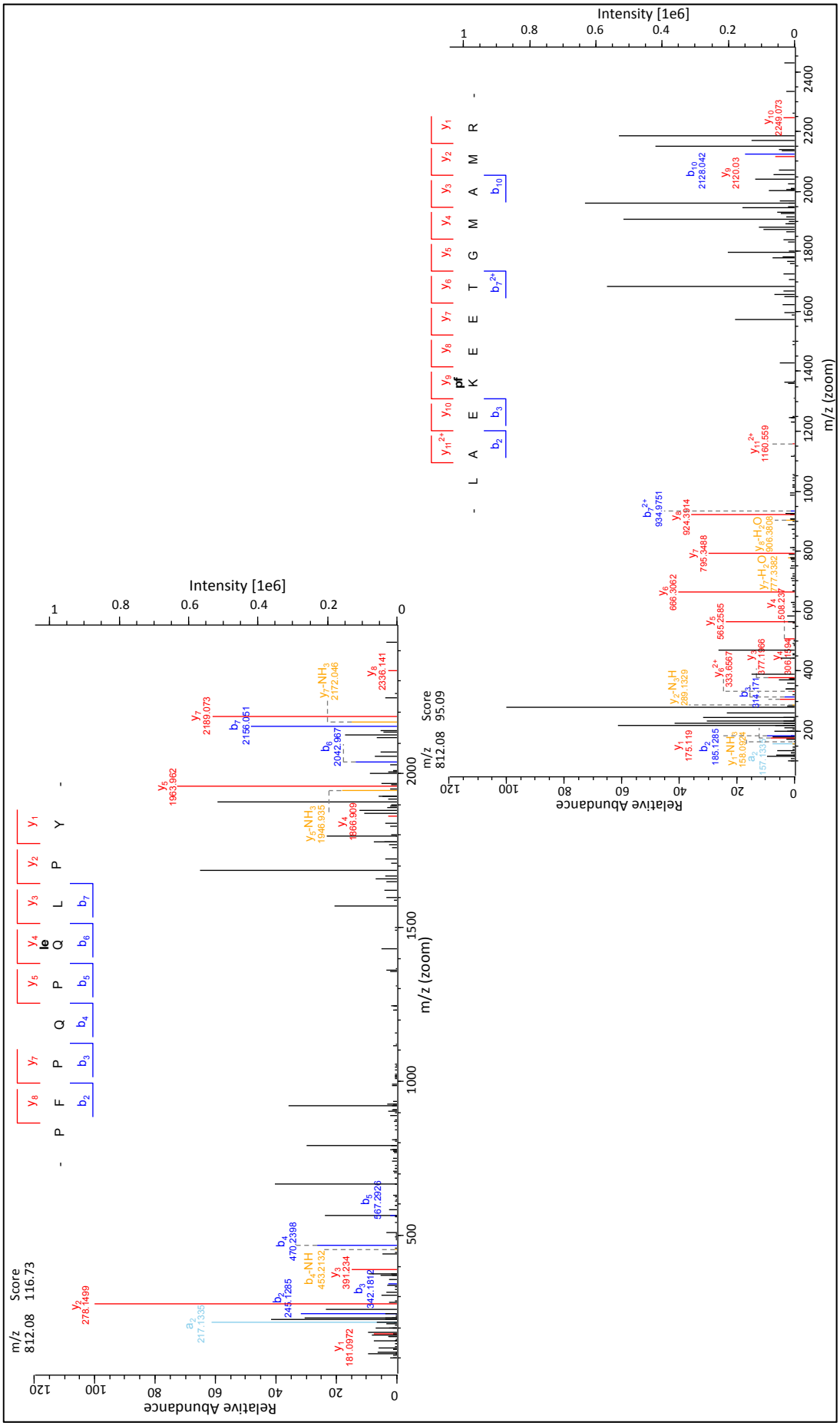

Figure 3. *Mass spectra of the isopeptide (m/z = 812.08 (3^+)) of the model peptide PepQ and the tryptic peptide LAEKEETGMAMR of TG2*

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4.7 Wheat grain proteins with impact on end-use quality and health attributes show significant responses on heat, drought and combined stresses

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Abstract

In the view of global climate change, heat and drought stress have become the most important limiting factors to crop productivity and food security. Beside this, there is some information about the effect of the elevated atmospheric CO_2 concentration to the yield, but not on the quality. These abiotic factors, like water deficits and high temperature generally occur at sensitive growth stages affecting functional properties of wheat. They are reflected in changes in the wheat yield; adversely affect the seed storage protein composition and the end-use quality. Wheat seed storage proteins are considered as key players in triggering different wheat related health disorders, containing immune responsive peptides (epitopes) that can cause immunological diseases. The main aim of our research is understand the shifts in the seed storage protein composition in different Hungarian bread wheat cultivars, caused by abiotic stress factors using proteomics and immunomics analyses. Performing climate chamber experiments and field trials we can evaluate the complex interpretation of specific heat- drought and combined abiotic-stress effects and monitoring the influence of elevated atmospheric CO_2 on the composition of these proteins. Characterisation of the protein content in the view of certain environmental conditions can provide a

powerful tool to predict the effect of climate change on the bread making quality and the accumulation of the harmful protein.

Introduction

In the past decades increased global temperature and climatic variability is resulting an increase of both the frequency and magnitude of extreme climate events [1]. Climate change can significantly affect environment, ecosystems, agriculture, and potentially affect crop production and quality [2]. Therefore cereals might have exposed to more than one stress event in the growing season [3].

With the rapidly changing climate combined abiotic stress factors can be detected, like high temperature, lack of water in the root zone, extreme transpiration and elevated atmospheric CO_2, which can cause significant changes in the yield, relative protein content, and composition of seed storage proteins, therefore affect the dough rheological parameters, and baking quality [4,5,6].

As one of the most important crops providing vegetal protein in human food, wheat plays a critical role in global food security. Storage proteins in wheat grain are the single greatest source of protein in the human diet. Maintaining grain quality under extreme climate conditions is crucial for human nutrition, end-use functional properties [7].

Wheat seed storage proteins represent 70-80 % of the full protein content, depends on species. They are key players in wheat-related diseases and health problems, like autoimmune reaction of coeliac patients. They form a typical protein profile of modern wheat genotypes, which confers to the dough its viscosity and elasticity. Bread quality is determined by the composition and molecular structure of gluten which turn controls the interaction of gluten subfractions during processing [8].

Abiotic stress factors and climatic extremities can cause changes in the protein profile of seed storage proteins, due to shifts in the composition and elimination of their balance.

The main purpose of the research was to observe the shifts and changes in the seed storage protein composition of preselected Hungarian bread wheat cultivars, in response to certain abiotic stress factors occurring at anthesis and post anthesis periods.

Climate chamber experiments and field trials were performed to evaluate the complex modelling of specific heat-, drought and combined abiotic stress effects and examine the impact of elevated atmospheric CO_2 on the wheat seed storage protein composition.

Interpretation of the protein content in response to certain environmental conditions can predict wheat acclimation, and effect of the climatic extremities on crop yield, relative protein content, bread making quality and accumulation of the harmful proteins in the future.

Performing field trials with experimental free air CO_2 enrichment (FACE) studies we can build up an accurate climate prediction model, which can underlies a specific breeding program.

Materials and Methods

Based on greenhouse heat and drought stress experiment out of 125 Hungarian bread wheat genotypes, derived from the Cereal Gene Bank of the Department of Plant Genetic Resources and Organic Breeding, Martonvásár, four wheat lines, a drought resistant, a drought sensitive, a heat resistant and a heat sensitive were selected.

Grain yield, and plant height were measured, thousand kernel weight of all samples were evaluated. Protein content was determined by near infrared spectroscopy (NIR) and Duma method. Grain samples were milled with Retsch Mixer Mill MM200.

SE-HPLC was performed to analyse the profile of seed storage proteins by determining the UPP% and Glutenin/Gliadin ratio. Harmful protein content was determined by using commercially available R5 (RIDASCREEN® Gliadin, R-Biopharm, AG, Darmstadt, Germany) and G12 (AgraQuant Gluten G12 Assay (4-200 ppm) ELISA assays.

Results and Discussion

In reference to the previously selected four bread wheat genotypes, according to the measured thousand kernel weight, combined drought and heat stress at anthesis and post anthesis period caused a significant reduction, 40-50 % kernel weight loss, in yield. However every genotypes shows increased protein content in response to combined stress effects, based on the UPP% and glutenin/gliadin ratio, due to changes in protein composition significant decrease can be found in quality of four of every genotypes (Fig. 1).

The results of commercially available R5 and G12 ELISA tests revealed that based on the climate chamber experiments characteristic changes occur in harmful protein content in response to drought stress and heat stress at anthesis and post anthesis period (data not shown). Further immunoanalyses are needed to evaluate different abiotic stress profiles.

Besides the selected bread wheat genotypes *Triticum monococcum* preliminary selected genotypes were examined by commercially available R5 and G12 ELISA assays as well. They show significant lower harmful protein content compare to the examined bread wheat genotypes.

Based on these findings further abiotic stress analysis and detailed examination of the immunogen protein content will be needed to determine the stability of the lower toxic protein content of the investigated einkorn genotypes.

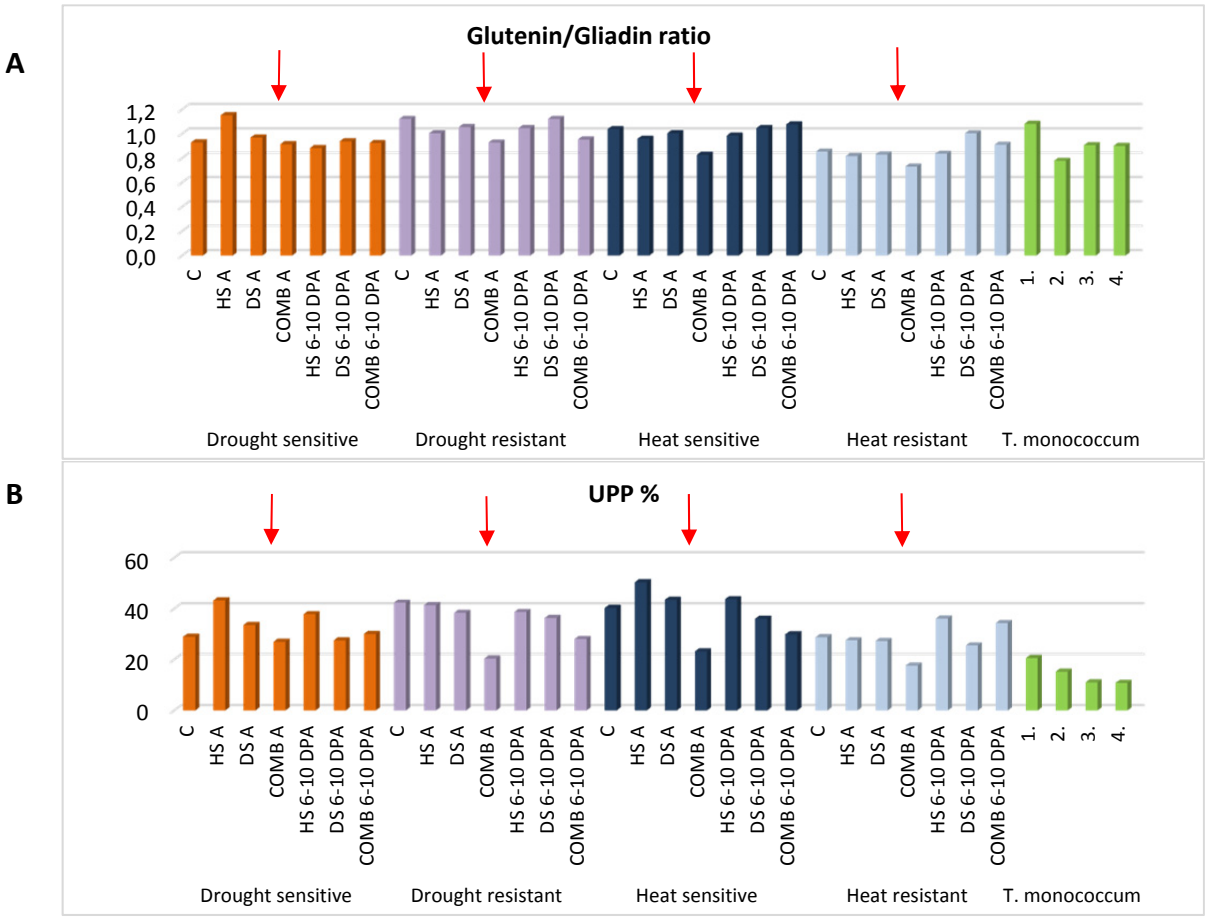

Figure 1. *Measured glutenin/gliadin ratio and UPP% (unextractable polymeric glutenin proteins) of four Hungarian bread wheat and preselected Triticum monococcum genotypes. C – control, DS A – drought stress at anthesis, HS A – heat stress at anthesis, DS 6-10 DPA – drought stress at 6-10 DPA, HS 6-10 DPA – heat stress at 6-10 DPA, COMB A – combined drought and heat stress at anthesis, COMB 6-10 DPA – combined drought and heat stress at 6-10 DPA*

Conclusion

Due to the increasing impact of climate change, abiotic stress factors such as higher temperature, lack of water, and elevated atmospheric CO_2 can cause significant changes in composition of seed storage proteins, therefore affect the dough rheological parameters, and baking quality.

Performing climate chamber experiments and field trials complex interpretation of specific heat, drought and combined abiotic stress effects can be evaluated, and the influence of elevated atmospheric CO_2 on the composition of these proteins can be monitored.

Observation of the influence of abiotic stress factors on protein quality and composition, and characterisation of the accumulation of harmful proteins provides us a powerful tool to predict the effect of climate change on wheat end use properties, and stability on response to certain environmental extremities. The results will lead us to

contribute developing an accurate climate modelling program, which focuses on complex factors of crop breeding.

Acknowledgement

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5 Clinical research reports

5.1 High enzymatic digestibility of *Triticum monococcum* gluten: evaluation of gluten stimulatory properties on T lymphocytes from coeliac gut mucosa

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Introduction

The ingestion of gluten proteins is the causative event triggering coeliac disease (CD) in genetically predisposed individuals [1]. Because of the high content of prolin and glutamine residues, gluten proteins are also termed as prolamines and represent the main storage proteins in wheat, barley, and rye [2]. The peculiar chemical composition confers to gluten proteins a high resistance to gastrointestinal digestion. As consequences, large proline/glutamine rich peptides reach the small intestine and trigger immune responses in patients with CD. Furthermore, the marked binding affinity to CD-associated HLA molecules, consequence of a deamidation mediated by tissue transglutaminase (tTGase), significantly increase the immunogenicity of gluten [4]. Several peptides from α-, ω- and γ-gliadins have been described, to date, as immunogenic in the great majority of both children and adults intolerant to gluten [5-7]. The genetic susceptibility is conferred by HLA class II genes encoding for DQ2.5 or DQ8 heterodimers [8]. However, although 30 % of general population carry the HLA DQ2.5/8 genes, it is estimated that only 1-3 % will develop CD in wheat consuming countries [9]. The incidence of CD is much higher in the first-degree relatives of coeliacs carrying the predisposing HLA-DQ genes, where the risk to develop CD has been estimated 10-fold increased (approximately 10 %) [10]. Gluten free diet is, currently, the only dietary therapy available for treatment of CD. However, many alternative therapeutic strategies, based on different methodologies, are, currently, under evaluation [11]. In the recent time an increasing interest has been addressed, in particular, to the identification of wheat species naturally devoid, or with a low content, of toxic gluten sequences [11-14], respectively suitable for treatment or prevention of CD.

Triticum monococcum (TM), or Einkorn, is an ancient diploid wheat of special interest to prevent CD. TM was the early wheat species to be cultivated by human beings, and remained almost genetically unchanged since 10.000 years ago. Of note, TM gluten proteins lack of a D-genome encoding the 33-mer, which is highly stimulatory of

gluten-reactive T cells in coeliacs, as it contains several overlapping T cell epitopes [13]. In addition, the good bakery and pasta-made, as well as nutritional, properties make TM a good candidate for the treatment or prevention of CD [15]. However, several studies, based on diverse experimental approaches, have showed conflicting results on the immune-toxicity of TM gluten proteins [12-14, 16-19].

We have examined the capability of TM gluten to stimulate T cells from coeliac patients after an extensive *in vitro* digestion, that reproduces the physiological gut degradation [13,14]. The digested gluten peptides from TM and soft wheat T*riticum aestivum* (TA), included as control, were used to in vitro challenge T-cell lines raised from intestinal mucosa of CD patients.

Materials and Methods

In vitro simulated extensive gastrointestinal digestion

Gliadin samples from the wheats TM (Norberto-ID331 cultivar) and TA (Sagittario cultivar) were enzymatically digested with sequential steps that included all proteases from gastric-duodenal-brush-border membrane sections, as previously reported [20-22]. Brush-border membrane enzymes (BBM) vesicles were isolated from pig jejunum according to Shirazi-Beechey *et al*. [23]. Briefly, each gliadin preparation was dissolved in 0.01 M chloride acid and incubated with pepsin (1:100, enzyme/substrate w/w ratio) for 30 min at pH 2.0. The pH was next adjusted to 7.0 (by adding phosphate buffer) and the gliadin mixtures were incubated with trypsin (1:100), chymotrypsin (1:100), elastase (1:100), and carboxypeptidase A (1:500) for 1 hour at 37°C. Thereafter, samples were two-fold diluted with 0.1 M sodium phosphate buffer pH 7.2, supplemented with BBM at 100 mU/100 mg of peptide amount, and incubated at 37°C for 6 hours (Fig. 1). Samples were stored at -80°C until further analysis.

Proteomic analysis of gastrointestinal resistant peptides and enzymatic deamidation

Gliadin proteins and peptides were identified using Nanoflow LC-ESI MS/MS analysis as described [22]. Gastrointestinal resistant peptides, released upon the extensive digestion, were fractionated by and HPLC and output peptide identifications were validated by manual inspection of MS/MS spectra. The MS/MS raw spectra were used to generate text files in mascot generic file format, and submitted to search engines using Protein Prospector (http://prospector.ucsf.edu). Gliadin proteolytic digests were next deamidated by tTGase (Sigma-Aldrich), as previously described [20]. Gliadin samples were incubated at 37°C for 4-hrs with TG2 (1:1 enzyme:substrate ratio) in 5 mM Tris-HCl buffer (pH 6.8), containing 5 mM CaCl2, 10 mM NaCl, and 10 mM dithiothreitol. Samples were next lyophilised and store at -20°C until the use on T cells.

Immunogenicity assay on intestinal T cells

T cell lines were established by repeated stimulations of intestinal cells isolated from three HLA-DQ2.5 adult CD patients, with autologous mononuclear cells and PT-gliadin digest. Growing cells were thereafter expanded in culture with phytohemagglutinin (PHA) and IL-2 and IL-15, as growth factors [12]. When in resting phase, expanded T cell cultures were assayed for recognition of deamidated gliadin enzymatic digests, as reported [12]. Briefly, $3x10^4$ T cells were co-incubated with $1x10^5$ allogeneic and HLA-matched Epstain Barr virus-transformed B cells (B-LCLs) at 37°C in complete medium (X-Vivo enriched with 5 % human serum-Gibco, Waltham, MA, USA) in 96-well plates. Gliadin enzymatic digests were used at 50 µg/mL. Cell supernatants (50 µL) were collected after 48 hours of cell incubation for the evaluation of INF-γ by a standard sandwich ELISA, as previously described [5].

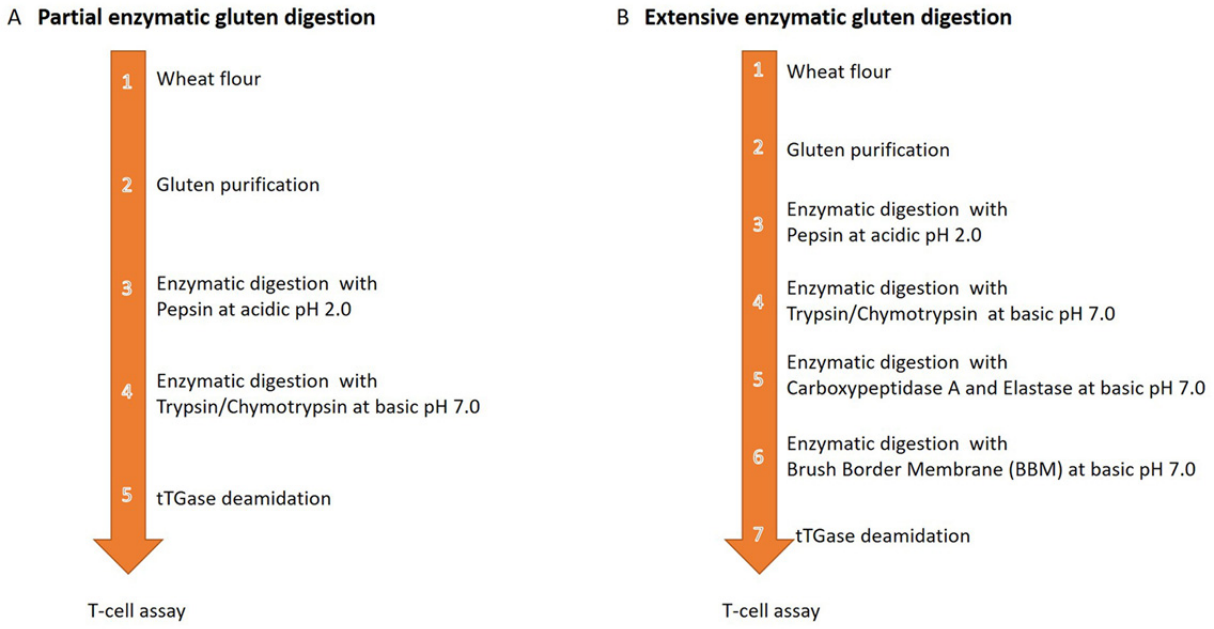


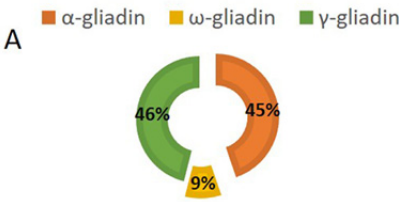
Figure 1. *Schematic description of in vitro digestive process of gluten proteins and functional assay on coeliac gut derived T cells. Digestion protocol widely used in most scientific studies since 1980 that include a partial degradation with pepsin/trypsin-chymotrypsin enzymes (**panel A**). Extensive in vitro digestion comprehensive of all gastro intestinal proteases, including the brush-border membrane enzymes (BBM) (**panel B**).*

Results and discussion

Gliadin extracts from ancient wheat species TM (Norberto-ID331 cultivar) and from soft wheat TA (Sagittario cultivar) were in vitro enzymatically degraded, according to an innovative protocol that aims to mimic the human physiological gastro-intestinal digestion (Fig. 1). The proteomic analysis of digestion resistant peptides demonstrated that gliadin proteins from the ancient wheat were more efficiently cleaved by GI-enzymes than gliadin proteins from the soft wheat (Fig. 2). As revealed by mass

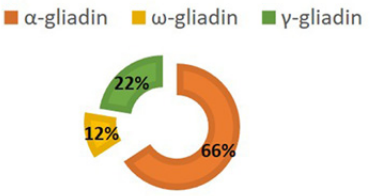
spectrometry analysis, a total of 76 peptides were identified as resistant to GI digestion in TM gliadins, by contrast, 240 peptides were detected after the extensive degradation of TA gliadin. Differences were also observed in the distribution of gastrointestinal resistant peptides among the gliadin protein families. A high percentage of α-gliadin (45 %) and γ-gliadin (46 %) peptides were found after the digestion of soft gliadin (Fig. 2A), on the other hand, from the hydrolysis of ancient wheat the α-gliadin (66 %) peptides were markedly prevalent (Fig. 2B). The sequence analysis of the gastrointestinal resistant peptides revealed that the α-gliadin 33-mer and 31-55 peptides, respectively eliciting and T-cell mediated and innate immune response, remained almost intact upon GI digestion of TA gliadin. Conversely, in the GI-digested TM gliadin only the 31-55 peptides remained undegraded. Fragments of DQ2.5-glia-α1a epitope (peptide 56-58 contained in 33-mer) and devoid of immunogenicity, were released upon the GI proteolysis in both TA and TM gliadins (Fig. 2C&D).

A



T. aestivum (Sagittario cultivar)	
Resistant peptides	241
α-gliadin	108
ω-gliadin	22
γ-gliadin	111

B



T. monococcum (Norberto-ID331 cultivar)	
Resistant peptides	76
α-gliadin	50
ω-gliadin	9
γ-gliadin	17

C

peptide	sequence
33mer:	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF
31-55:	LGQQQPFPPQQPYPQPQPFPSQQPY
Fragment of 56-68:	QLQPFPQPQ QLQPFPQP LQPFPQPQ QPFPQ

D

peptide	sequence
31-55:	LGQQQPFPPQQPYPQPQPFPSQQPY
Fragment of 56-68:	QLQPFPQPQ QLQPFPQP LQPFPQPQ QPFPQ

Figure 2. *Profile of gliadin peptides resistant to the extensive in vitro digestion of gliadin from soft wheat Triticum aestivum (Sagittario cultivar) (panel A&C) and from ancient wheat Triticum monococcum (Norberto-ID331 cultivar) (panel B&D). Peptide identification was obtained by the mass spectrometry analysis.*

Overall, these data demonstrated that gliadin proteins from ancient and modern grains have a dissimilar sensibility to GI degradation, that reflects the diverse genotype of two wheat species and the different chemical structure of gliadins. In addition to the proteomic characterisation, we next focused on the evaluation on how the extensive digestion may affects the immunological properties of the two wheat species. Of note,

previous studies reported conflicting results regarding the potential immune safety of TM wheat for CD patients [12-19]. In order to have a clearer comprehension of the immunogenicity of the ancient wheat, we evaluated the capability of TM gliadin to activate coeliac gut T cells upon the physiologic GI proteolytic process. The detection of the inflammatory cytokine INF-γ was used as sensitive probe to evaluate the immune stimulatory potential of gliadins [5]. INF-γ production was assessed in T cell lines previously obtained from the intestinal mucosa of three CD patients and highly reactive to undigested TA gliadin [5,18,19]. In Fig. 3, the immune activation of gut T cells in response to the GI-digested gliadin from both TA and TM wheats are shown. Interestingly, we found that in all CD patients, the INF-γ production elicited by TM gliadin was markedly reduced compared to the amount detected in response to TA gliadin in all the TCLs assessed.

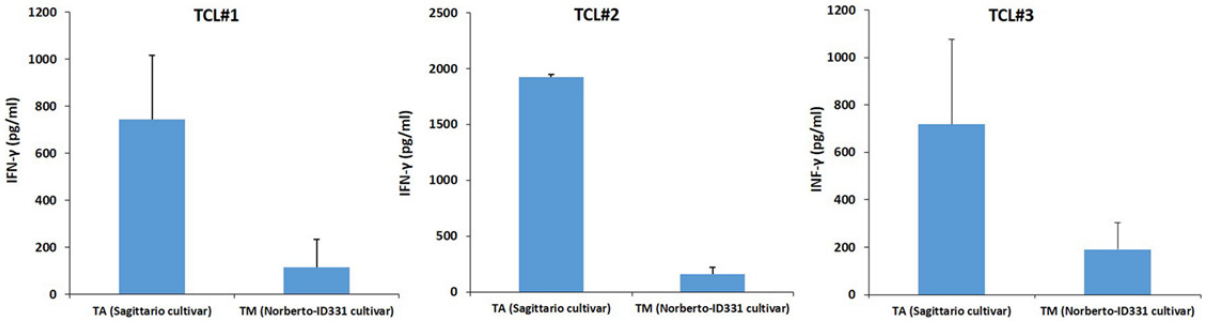


Figure 3. *INF-γ responses obtained after* in vitro *stimulation of gliadin-reactive T cell lines (TCL) established from intestinal mucosa of three coeliac patients. Gut T cells were* in vitro *stimulated with the extensively digested gliadin from the soft wheat* Triticum aestivum *and ancient wheat* Triticum monococcum*. Gliadin GI-digests were assayed at 50 µg/mL. The INF-γ production was evaluated in cell supernatants after 48 hours of culture by enzyme-linked immunosorbent assay (ELISA).*

These findings, obtained with coeliac gut T-cell assay, indicated that TM gliadin is still toxic for coeliac patients, however after an *in vitro* extensive digestion retained a reduced immunogenicity if compared to TA gliadin. The data on T cell reactivity profile are in line with the proteomic analysis demonstrating a higher sensibility of TM gliadin to be hydrolysed by gastrointestinal proteases that resulted in a reduced number of immunogenic peptides.

Conclusions

In conclusion, we used an innovative *in vitro* protocol that mimic the physiological gastrointestinal protein digestion, to evaluate the immunological properties of gluten from the diploid wheat *T. monococcum*. Compared to gluten from the modern wheat, such as the hexaploid *T. aestivum*, a low number of peptides remained undigested upon the extensive digestion with gastro-intestinal proteases, including those of brush-border membrane. The reduced amount of released peptides well correlated with the

disappearance of the most immunogenic gliadin sequences and a low capability to activate a T cells from coeliac gut mucosa.

Based on these findings, we suggested that the introduction of a wheat markedly sensible to the GI digestion, might be of benefit for those patients at high risk to develop CD, such as first-degree relatives of CD patients that carry the HLA genetic predisposition. It would be of great interest to assess whether a prolonged *T. monococcum* containing diet, may result in a reduction of CD incidence in at risk subjects.

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5.2 Normalisation of serology in potential coeliac disease subjects does not invariably lead to a full normalisation of intestinal inflammatory signs

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Introduction

Coeliac disease (CD) is an immune-mediated systemic disorder elicited by gluten and related prolamins in genetically susceptible individuals, characterised by the presence of a variable combination of clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy [1]. The severity of the intestinal damage can range from villous atrophy (VA), typical of the overt disease, to minor or absent histological alterations. The term potential coeliac disease (PCD) has been coined to identify a subset of patients who present an increase in IgA anti-tissue transglutaminase antibodies (anti-TG2) in the blood, but a normal intestinal architecture [2]. Among PCD, some show a completely normal mucosa (Marsh0), others display an increase in the numbers of intraepithelial lymphocytes (Marsh1). The natural history of PCD has not been fully elucidated yet. Nevertheless, in our longitudinal cohort, we observed that around one third of patients developed full-blown disease with villous atrophy over a 9 years follow-up, while 67 % of them showed a persistently normal duodenal architecture, despite the positive serology [3]. Among those who did not develop tissue damage, about one third showed persistently elevated anti-TG2 IgA level, another third showed fluctuant levels of anti-TG2 with transiently negative values, while the rest of them showed a persistent normalisation of antibodies titers over the course of the follow-up [3]. Why only one third of PCD patients develop full-blow disease remains to be assessed. Whether it is only a matter of timing and all of them will eventually develop full-blown disease during the course of their lives will require a longer and attentive follow-up. On the other hand, why some individuals who displayed positive coeliac-specific serology, will then show a full normalisation of the antibody titers and never evolve toward full-blown disease remains unexplained. In this study we aimed at investigating the histological features of this last category of PCD patients.

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Patients and methods

In our cohort of over three-hundred PCD patients we identified 69 subjects who became persistently seronegative during follow-up (69/330, 21 %). All of them displayed positive anti-tissue tranglutaminase2 (anti-TG2) and/or anti-endomysium (EMA) IgA antibodies in at least two different determinations and showed a normal small intestinal architecture at the time of diagnosis. We considered as "seronegative" each subject persistently showing negative serum titers of anti-TG2 and EMA antibodies for at least two years after the last positive detection. The detection of positive titers of either one of these two antibodies at any time during follow-up was considered an exclusion criterion. None of these subjects had a concomitant IgA deficiency, thus there was no need to consider IgG class antibodies and/or anti-deamidated gliadin (DGP) antibodies. Anti-TG2 antibodies and EMA were detected by ELISA and immunofluorescence, respectively.

In each patient, esophagogastroduodenoscopy was performed and five biopsies were taken from the duodenum, including one fragment from the bulb and four from the distal duodenum. Moreover, 15 out of 69 seronegative PCD patients underwent a second endoscopy of the upper gastro-intestinal tract, in most cases to investigate the onset of clinical symptoms potentially related to CD. Thus, we collected a second biopsy at time of normalisation of anti-TG2 IgA serum antibodies.

Four out of five fragments, including the one from the bulb, were fixed in 10 % formalin, embedded in paraffin, and then stained with hematoxylin. The histological and morphometrical analysis was performed by light microscopy. A villous height to crypt depth ratio (V:H) above 2 was considered normal [4]. The Marsh scoring was assessed upon counting the number of lymphocytes infiltrating the small intestinal epithelium (IELs): the presence of less or more than 25 IELs per 100 enterocytes defined a Marsh score of 0 or 1, respectively. All fragments were scored according to Marsh criteria by an expert pathologist who was blinded to any serology results, however the assigned score was based on the fragment with the worst histopathological picture. One fragment for each patient was included in an optimal cutting temperature (OCT) compound (Killik, Bio-Optica, Milan, Italy), stored in liquid nitrogen and subsequently used for immunohistochemical staining using anti-$CD3^+$ and anti-TcR-$\gamma\delta^+$ antibodies, as previously reported [5]. The number of stained $CD3^+$ and TcR-$\gamma\delta^+$ IELs per millimetre of epithelium was determined. Cut-off values of 34 $CD3^+$ IELs and 3.4 TcR-$\gamma\delta^+$ IELs/mm of epithelium, were used to discriminate normal vs increased infiltration. In addition, intestinal anti-TG2 IgA antibody deposits were evaluated by double immunofluorescence on five-micron OCT sections and evaluated using a fluorescent microscope (Axioskop2- plus; Zeiss MicroImaging Inc, Milan, Italy). The intensity of fluorescence was assessed based on the staining intensity and distribution. Statistical analysis was performed using GraphPad Prism4 for Windows, version 4.03. Data were compared by Mann-Whitney or χ^2 test. A p-value below 0.05 was considered to be statistically significant.

Results

In our cohort of 330 PCD patients followed-up over 10 years 69/330 (21 %) became seronegative despite staying on a gluten containing diet (GCD). Most of them (59/69, 85 %) became seronegative within the first 24 months of follow-up. Each subject defined as PCD received a small intestinal biopsy at time of first appearance of IgA anti-TG2 antibodies in the serum that showed a normal mucosa (Marsh0) or slightly infiltrated epithelium, without any sign of crypts hyperplasia and/or villous blunting (Marsh1). Moreover for 15/69 seronegative PCD patients we had the chance to collect a second biopsy at time of normalisation of serum anti-TG2 antibodies. Comparing the histological features of the biopsies at time of diagnosis (t0) and upon normalisation of coeliac-specific serology (t1), no significant change in Marsh scoring was found upon anti-TG2 normalisation. In particular, Marsh score remained unaltered for 9/15 (60 %), while it improved from M1 to M0 in 4/15 (26.7 %) subjects. Unexpectedly, it changed from M0 to M1 in 2/15 (13.3 %) subjects.

Immunohistochemical analysis revealed a reduction in the number of TcR-$\gamma\delta^+$ IELs, but not of the number of $CD3^+$ IELs upon normalisation of anti-TG2 antibodies serum levels. A moderate, although not significant, decrease of the TcR-γδ/CD3 ratio was also observed. Interestingly, even though most patients showed a decrease in the numbers of $CD3^+$ (60 %) and/or TcR-$\gamma\delta^+$ (66.7 %) IELs, more than 30 % of them retained signs of inflammation in the intestinal mucosa despite serology normalisation and around 20 % of them showed even an increase of both TcR-$\gamma\delta^+$ and $CD3^+$ IELs.

Double immunofluorescence analysis performed to highlight the presence of intestinal anti-TG2 antibodies' deposits revealed that most patients maintained a local production of CD-specific antibodies in the duodenal mucosa, despite the normalisation of their serum levels. However, even though 54 % of subjects retained an intestinal production of anti-TG2 antibodies, a significant lower rate of them (69.2 % at t0 vs 30.8 % at t1, χ^2 test p=0.05) showed a high intensity (>2) of such deposits.

Altogether, these data suggest that despite the disappearance of serum anti-TG2 antibodies, a significant proportion of PCD patients retained inflammatory signs in the small intestinal mucosa, including $CD3^+$ IELs (33 %), TcR-$\gamma\delta^+$ IELs (55 %), TcR-γδ/CD3 (46 %) above the cut-off values and duodenal production of anti-TG2 antibodies (50 %).

Conclusions

This preliminary study provides evidence that a subset of PCD patients can develop a persistent normalisation of anti-TG2 serum titres over time, despite being on a GCD. Notably, even though the coeliac-specific serology normalises over time, most of these patients continued displaying some signs of small intestinal inflammation or the persistence of a local abnormal gluten-dependent mucosal immune response. More specifically, independently from the level of infiltration of the small intestinal epithelium at the time of diagnosis, most patients showed still abnormal numbers of

TcR-$\gamma\delta^+$ IELs upon normalisation of anti-TG2 antibodies, suggesting that some alteration at the level of the epithelium might be still present and responsible for the recruitment or the persistence of a pool of TcR-$\gamma\delta^+$ IELs even in the absence of peripheral signs of an ongoing gluten-dependent humoral response. What these factors are remains to be assessed. Furthermore, the persistent production of intestinal anti-TG2 antibodies in more than half of these seronegative PCD patients suggests that a gluten-dependent immune response can still be ongoing locally at the level of the duodenal mucosa. Notably, the magnitude of this response is significantly lower upon normalisation of serology, suggesting that despite the persistency of an inflammatory response in the duodenum, its magnitude decreases in parallel with the downregulation of the humoral gluten-specific immune response. Whether these mucosal inflammatory hallmarks of CD will persist on a GCD diet despite a prolonged antibody normalisation in the serum, or whether they require longer time to disappear, remains to be addressed. A longer follow-up will allow clarifying this aspect. Accordingly, we suggest that patients who showed, during their lifetime, positive CD-associated serology require a careful follow-up even upon seroconvertion (anti-TG2 and/or EMA normalisation).

Together with a series of previously reported evidences, our results contribute to support the idea that PCD is a heterogeneous population. It will be pivotal to identify the different subsets of PCD as well as their clinical features. Furthermore, the identification of biomarkers that can predict their natural history would be pivotal and will help implementing the most appropriate management for these patients.

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5.3 Study of cell death in duodenal mucosa in active coeliac disease

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Introduction

Coeliac disease (CD) is a multifactorial immune-mediated disease that develops in genetically susceptible individuals after ingestion of a group of proteins derived from wheat, barley and rye, commonly named gluten. CD is a chronic enteropathy characterised by severe changes in the histology of proximal small intestine mucosa. These changes include massive loss of enterocytes and villi, and crypt hyperplasia [1].

The increased enterocyte death has been explained by an uncontrolled apoptosis induction due to excessive activation of intraepithelial lymphocytes (IELs) [2,3]. Apoptosis has been considered as the main pathway of enterocytes death, however this mechanism has been only partially studied [4].

The Fas-Fas ligand (FasL) system has been considered the major pathways in inducing enterocyte apoptosis [5]. FAS and FASL belong to the Tumor Necrosis Factor (TNF) family. While FAS is expressed constitutively in different tissues it can also be induced by proinflammatory cytokines, FASL is expressed mainly on activated T cells and natural killer cells.

A different mechanism for enterocyte killing involved the recognition of stress signals (MICA) [6] on enterocytes by activating receptor (NKG2D) presents in intraepithelial cells has also been reported [7].

Pyroptosis is a lytic form of cell death distinguished from apoptosis produced as consequence of the activation of the inflammasome platform. Particularly, caspases 1/11 cleave Gasdermin D (GSDMD) protein to generate a N-terminal domain, which forms a pore after location on the plasma membrane [8]. Through these pores, small molecules as some proinflammatory cytokines (IL-1β) and intracellular contents, are released [9,10]. Dysregulation of pyroptosis promoted by microbial infection and danger signals is often associated with excessive inflammation leading to a number of inflammatory diseases [11,12].

The aim of this work was to characterise the cell death pathways in intestinal mucosa in CD enteropathy.

Materials and methods

Patients

Duodenal biopsies were collected from paediatric and adult patients during the routine procedure for CD diagnosis in the Gastroenterology Units of Hospital Sor María Ludovica (paediatric patients) and Hospital San Martín (adult patients).

CD diagnosis was based on histological findings in duodenal sections, serological analysis and clinical signs. Individuals in the control group were subjected to endoscopic study for other clinical conditions (mainly dyspepsia or diarrhoea). All of them presented, at the time of evaluation, negative serology for CD markers and normal duodenal histology. Ethic committees from Public Health Institutions approved this work.

Experiments

Expression of caspase 1, 3, 8 and Gasdermin D (GSDMD) were assessed by Western blot (WB) in protein extracts from whole biopsy. Proteins were separated by 12.5 % or 15 % polyacrylamide gels on SDS-PAGE. WB analysis was performed by incubating with primary specific antibodies: β-Actin (Abcam, cat ab8227), Caspase 1 (Santa Cruz, cat sc-56036), Caspase 3 (CellSignaling, cat 9662), Caspase 8 (CellSignaling, cat 9746) and GSDMD (Santa Cruz, cat sc-376318). Secondary anti-mouse IgG-HRP antibody conjugated (Abcam, cat ab6789) or anti-rabbit IgG-HRP conjugate (BioRad, cat #1706515) were used. Visualisation of antibody reactivity was performed by chemiluminescence, using a commercial reagent (GE Healthcare, cat RPN2232), and they were revealed with Romek photographic reagents (Gamaxa SRL, Argentina) according to the manufacturer's instructions. Image analysis for relative quantification of optical density was performed with ImageJ software. The optical density in the band of interest corresponding to the protein under study was quantified and normalised to the value of β-actin.

TUNEL reaction and immunofluorescence microscopy (IFI) analysis were performed on sections of paraffin-embedded tissues. For TUNEL reaction The Dead End Fluorometric TUNEL system kit (Promega) was used, following the manufacturer's instructions. Cell count on fluorescence images was performed using the ImageJ software. The result was expressed as the number of $TUNEL^+$ cells per μm^2 of *lamina propria*. Images were obtained using a Leica SP5 confocal microscope. The cell density was calculated as the number of positive cells for each marker per unit area in appropriate regions of tissue sections.

Statistics

Statistical analysis was performed using the GraphPad Prism 6.0 program. All determinations between controls subjects and active CD patients were performed using unpaired T-test with 95 % confidence, values of $p < 0.05$ were considered significant.

Results and discussion

Using different markers and techniques, we aimed to characterise the cell death process in the duodenal mucosa of untreated CD patients. First, we studied the canonical apoptosis pathway, by analysing cell death by TUNEL reaction, caspase 8 and caspase 3 expression (Tab. 1).

Table 1. *Evaluation of cell death pathways in duodenal mucosa*

	Active CD			Controls			Stats	Method
	n	**Median**	**SD**	**n**	**Median**	**SD**		
TUNEL$^+$	6	9.66e-05	2.3e-005	6	2.33e-05	2.3e-005	Test T unpaired (*p <0.05)	(cells per μm^2) Fluorescence microscopy
Active Caspase 3	7	40.07	14.42	6	2.821	0.82	Test T unpaired (**p<0.002)	(% Relative Units) Western Blot
Caspase 3	4	19,450	2,250	5	5,750	8,121	Test T unpaired (*p<0.03)	(Relative Units) Fluorescence microscopy
Caspase 8	4	27,700	8,279	5	4,000	7,895	Test T unpaired (*p<0,05)	(Relative Units) Fluorescence microscopy
Caspase 1	6	48,750	33,806	7	16,500	12,209	Test T unpaired (*p<0.03)	(Relative Units) Fluorescence microscopy
GASDMD	4	61,500	14,949	4	7,750	2,302	Test T unpaired (*p<0.03)	(Relative Units) Fluorescence microscopy

The TUNEL reaction, which represents an advanced stage in cell death, showed a higher number of TUNEL$^+$ cells of duodenal *lamina propria* in CD patients compared to healthy controls. The massive number of *lamina propria* TUNEL$^+$ cells scattered in the tissue is intriguing. Identification of lineage of dead cells is in progress.

To evaluate the extrinsic pathway of apoptosis, caspase 3 and 8 were studied by immunofluorescence analysis. Mean fluorescence intensity was higher for caspase 3 and 8 expression in duodenal samples of CD patients.

Since this evaluation cannot identify the active fraction of this protein, WB analysis was performed to determine the cleaved caspase 3. WB analysis on total protein extracts from whole duodenal biopsies showed increased levels of active caspase 3 in samples from CD patients.

Finally, to assess whether pyroptosis may occur in the duodenum as part of the pathogenic process, gasdermin D (GSDMD) was evaluated by immunofluorescence assay on sections of duodenal samples. Levels of GSDMD were found statistically higher in untreated CD patients compared with disease controls (Tab. 1).

Conclusion

In this study, we used immunofluorescence techniques and WB analysis to evaluate critical components of cell death pathways in duodenal samples from untreated CD patients and disease controls.

A high number of $TUNEL^+$ cells, together with increased caspase 8 and caspase 3 levels in both epithelium and *lamina propria* cells were found in duodenal samples of active CD patients. These findings are in agreement with previous descriptions about apoptosis as a cell death pathway in the mucosa of untreated CD patients. In addition, we showed activation of inflammasome/caspase 1/gasdermin D axis which suggest that pyroptosis may also occur in this tissue.

In conclusion, we found that multiple pathways associated with cell death and inflammation are activated in the small intestine of untreated CD patients. These findings suggest that apoptosis, as well as pyroptosis, may occur jointly in the enteropathy.

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5.4 Deamidated gliadins worsen immune reaction in food allergies

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Introduction

A food allergy, or hypersensitivity, is an abnormal response to an allergen that is triggered by the immune system. The prevalence of food allergies varies from 4 to 8% in children and from 1 to 5% in adults [1,2] and is constantly on the rise, making food allergies a topical issue. Wheat is a major component of the human diet, particularly in Western countries. Among food allergies, wheat allergy is predominant with an estimated prevalence of 0.4% based on oral challenge experiments in American and European populations [2,3]. Proteins derived from wheat grain have been implicated in food allergies. In both children and adults, wheat allergy induces a variety of symptoms: atopic eczema, atopic dermatitis, chronic urticaria, anaphylactic shock or exercise induced anaphylaxis [4,5].

Wheat is composed of two fractions: the insoluble fraction and the soluble fraction. The insoluble fraction is called gluten, and its proteins can be divided into gliadins and glutenins. Wheat, particularly the gluten, contains a large variety of proteins that can be allergens. Gluten has the particularity of forming a thick gel with great visco-elastic properties. Gluten is largely used in the food industry, mostly because of its structure. Indeed, it improves the texture and increases the protein quantity in food products. However, its solubility restricts its utilisation as a food ingredient. To counteract this problem, various processes such as deamidation (also called acid-hydrolysis), have been used to improve its solubility. However, these processes can change the structure of the proteins, and it has been shown that some processes produce new allergens [6].

Murine models constitute good tools to improve our understanding of allergic sensitisation mechanisms and symptom elicitation [7,8]. The allergic response to wheat proteins observed after a food allergy protocol is mostly orchestrated by Th2 lymphocytes. The sensitisation to some acid-hydrolysed wheat proteins increases the production of IgE and IgG1 and the secretion of IL-4 and IL-5 by splenocytes [7]. In the same way, sensitisation to deamidated gliadins induces the secretion of IgE, IgG1 and IL-4 [8].

Dendritic cells (DCs) are a key player in the establishment of oral tolerance and in the induction of specific immune responses. In particular, specific populations of DCs in the lamina propria are responsible for sampling soluble antigens in the intestinal lumen

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by extending their dendrites through the tight junctions of the intestinal epithelium and transporting them to mesenteric lymph nodes (MLNs). This process is necessary for the establishment of a specific immune response resulting in the activation of T and B cells but also in the generation of regulatory T cells [9,10].

Even if the allergic response is well-documented, the exact mechanisms of the immune response toward different forms of the same protein are still unclear. Indeed, how the industrial processes modifying wheat proteins impact the immune mechanisms is unknown. Our work aimed to study the effect of two forms of gliadins, native and deamidated, on the sensitisation phase and the elicitation phase in a murine model of food allergy. The immune response toward native gliadins and deamidated gliadins was studied at three different time points: after the first sensitisation, after the second sensitisation and after the oral challenge by gavage [11].

Materials and methods

Animal model

Balb/c mice from Janvier, France were used and housed in a ventilated cage system. The protocol was approved by the Ethics Committee on Animal Experimentation of Pays de Loire (CEEA) under the authorisation number 4049. Wild-type, 4-week-old balb/cmice were sensitised to wheat native gliadins (NG) or deamidated gliadins (DG) by two intraperitoneal injections of 10 μg in 100 μL of phosphate-buffered saline (PBS) plus 100 μL of aluminium hydroxide and separated for 10 days. After one week, mice were orally administered 20 mg of NG to induce the allergic reaction. Control mice were sensitised intraperitoneally with aluminium hydroxide only and challenged with PBS.

Gliadin preparation

Crude gliadins were extracted from wheat flour (Hardi cultivar) using the sequential procedure. Briefly, the albumin–globulin fraction of the flour was removed by several washings with saline buffer, and the flour was suspended in 70% ethanol for 1 h at room temperature (RT) to solubilise the gliadins. After centrifugation (20 000 × g for 20 min at 41 °C), the supernatant, that is the whole NG extract, was collected and freeze-dried. Deamidation was carried out by dispersing 20 mg of NG in 1 mL of 0.1 N hydrochloric acid. The temperature was increased to 90 °C and maintained at this level for 1 h. The reaction was stopped by neutralizing with 0.1 N NaOH. The resulting mixture was dialysed for 5 days against water (changed every day) and centrifuged to recover the supernatant. This soluble material was then freeze-dried (DG).

Measure of ear thickness

Just before the food allergy challenge, the ear thickness of the mice was measured using a digital micrometre. One hour after gavage, the ear thickness was measured a

second time. The results are expressed as the difference between the ear thickness measured after the challenge and the ear thickness measured before it.

Immunoglobulin assay

Blood was removed by cardiac puncture 1 h after the challenge and centrifuged at 3000 rpm for 15 min. The quantification of gliadin-specific IgE was assayed on serum samples by indirect ELISA as previously described [11,12,13].

Histology

One centimetre pieces of jejunum were extracted from mice, fixed in 4% paraformaldehyde (PFA) for at least 48 h, embedded in paraffin and cut and stained with hematoxylin eosin for morphological studies and inflammatory scoring. Histological images were taken with an Axio Imager M1 microscope and an Axiocam HRc (Zeiss). Sections were analysed using ImageScope for image acquisition (Aperio Technologies, Inc.), and a cell type count for goblet cells and leukocytes was performed with 10 villi per mouse. Grading of intestinal inflammation was determined in a blinded fashion as previously described [14,15]. The total histological score represents the sum of all features evaluated and thus ranges from 0 to 10.

Isolation of cells and flow cytometry

Mesenteric Lymph Nodes (MLNs) were collected from mice and ground. In all, 1.10^6 cells were transferred to a 96-well round-bottom plate and stimulated for 5 h with 50 ng/mL of phorbol-12-myristate-13-acetate and 1 μg/mL of ionomycin (Sigma-Aldrich) together with brefeldin A (10 μg/mL; BD Bioscience). Cells were stained with surface markers (CD3-FITC, CD4-Alexa 405, CD25-PE-Cy7, CD11c-BV510, MHCII-APC-Cy7, SiglecH-PE, CD11b-APC, Ly6C-APCH7, CD103-BV4, CD68-BV4, Ly6G-PE-Cy7, B220-PerCP5.5, BD Biosciences) in the presence of Fc blocker mouse CD16/CD32 antibody (BD Biosciences). For intracellular staining, the cells were fixed and permeabilised using a Cytofix/Cytoperm kit (BD Biosciences) and stained with IL-4-PE, Foxp3-APC, IFN-y-APC, RORγt-PE, or IL-17-APC-H7 (BD Biosciences). Cells were analysed on a Canto II flow cytometer (BD Biosciences). Data were acquired using Diva 8.0 software and analysed with FlowJo X (TreeStar).

Statistical analysis

Data were analysed using GraphPad Prism 6.0 (La Jolla, CA, USA). Values were expressed as the mean ± SEM and compared using one-way ANOVA. In the case of a significant ANOVA, means were compared using the Mann–Whitney U test, and the results were corrected by Dunn's multiple-comparisons test. A p-value of less than 0.05 was considered significant.

Results and discussion

To investigate whether development of allergic sensitisation and elicitation to different wheat gliadin proteins is different, mice were sensitised and challenged with NG or DG, using a validated protocol for food allergy (Fig. 1A) [14]. To characterise the allergic symptoms, we measured the ear thickness with a micrometre after the first sensitisation, after the second sensitisation, and after the oral challenge. The ear thickness measured after each one of the sensitisations was the same for all the groups, supporting the notion that it is the oral challenge that really triggers the allergic response (data not shown). Our results show a significant increase in ear thickness after the oral challenge for the mice sensitised with the deamidated form of gliadins compared to mice sensitised with NG (101 vs 80 µm, $p < 0.05$) (Fig. 1B). The same results were observed when measuring the level of wheat-specific IgE in the blood of mice after the oral challenge (Fig. 1C). In fact, mice sensitised and challenged with deamidated gliadins had higher levels of specific IgE compared to native gliadin allergic mice (22 vs 14 mA.U, $p < 0.001$). At the tissue level, we assessed tissue inflammation by hematoxylin-eosin staining of jejunum fractions (Fig. 1D). In line with the ear thickness increase and specific IgE levels, mice allergic to deamidated gliadins exhibited stronger alterations of the intestine compared to mice allergic to NG (4.13 vs 5.3, $p < 0.01$) (Fig. 1E). Taken together, these results demonstrate that the sensitisation and challenge with the deamidated form of gliadins strengthened the allergic response [11].

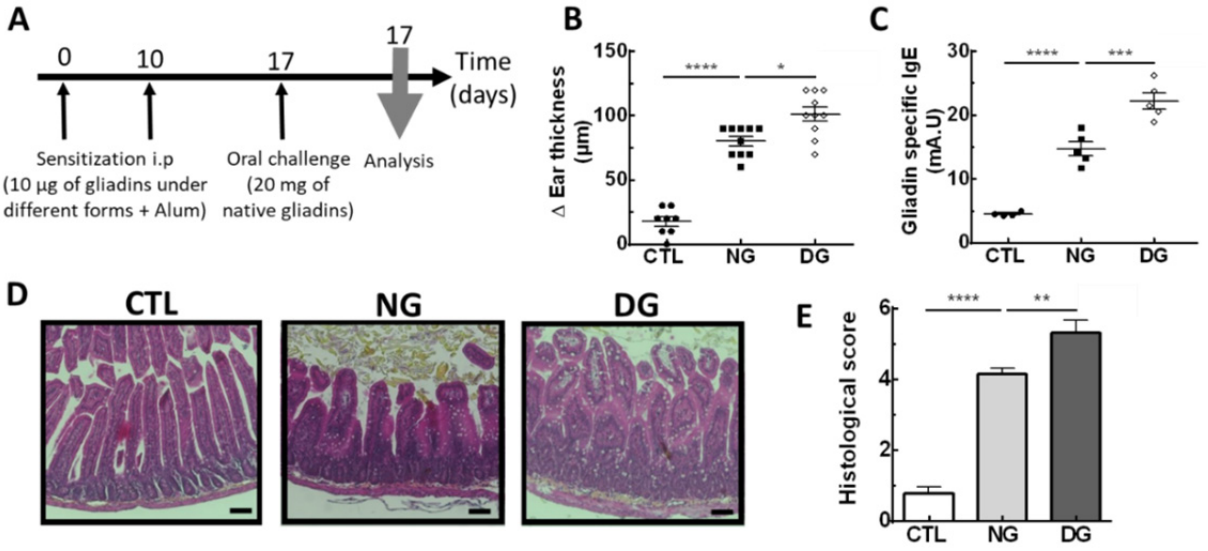

Figure 1. The deamidated forms of wheat gliadins amplify the allergic symptoms. *A) Mice were sensitised by intra-peritoneal injection of either DG or NG and then challenged with NG by gavage. B) Measures of the ear thickness after the oral challenge. C) Measures of wheat-specific IgE in serum of the three groups of mice after the challenge. D) Hematoxylin-eosin stained jejunum sections from the CTL and mice sensitised to NG or DG. E) Histological score calculated from jejunum sections according to inflammation, crypt damage, and ulceration in three groups of mice. Data represent the mean ± SEM (n = 8–10 animals per group); *p < 0.05, **p < 0.01, and ***p < 0.001, ****p < 0.0001. Scale bar = 100 µm.*

Our results are consistent with the observations in humans. In fact, the symptoms of patients allergic to deamidated wheat proteins (anaphylactic shock, exercise-induced anaphylaxis (ElA), urticaria) are often more severe than symptoms of patients allergic to wheat (more often gut irritation, allergic rhinitis, asthma, and atopic eczema/dermatitis syndrome (AEDS) than EIA and anaphylactic shock) [16,17]. Our results demonstrate that deamidation induced by industrial processes provokes an increase in allergic severity potential, as it has been suggested for peanut allergens [18]. For peanuts, the allergic potential modification might be linked to protein structure.

To characterise the immune response activated during sensitisation, we analysed cell infiltration in the MLNs after the second sensitisation (Fig. 2). In line with our previous results, the first sensitisation was not effective at inducing an immune response as there was no increase in dendritic cell frequency in MLNs (data not shown). In contrast, after the second sensitisation with DG, an increase of two- to threefold was observed in the frequency of DCs in MLNs compared to native gliadin-sensitised mice (Fig. 2A). The NG group did not show an increase in dendritic cell frequency compared to control mice. Transcriptomic, phenotypic, and functional analyses indicate that human and murine DCs can be divided into three subsets: two subsets of conventional DCs (cDC) called cDC1 and cDC2, and a third lineage of plasmacytoid DCs (pDCs). Each subset is unique and has distinct functional specificities

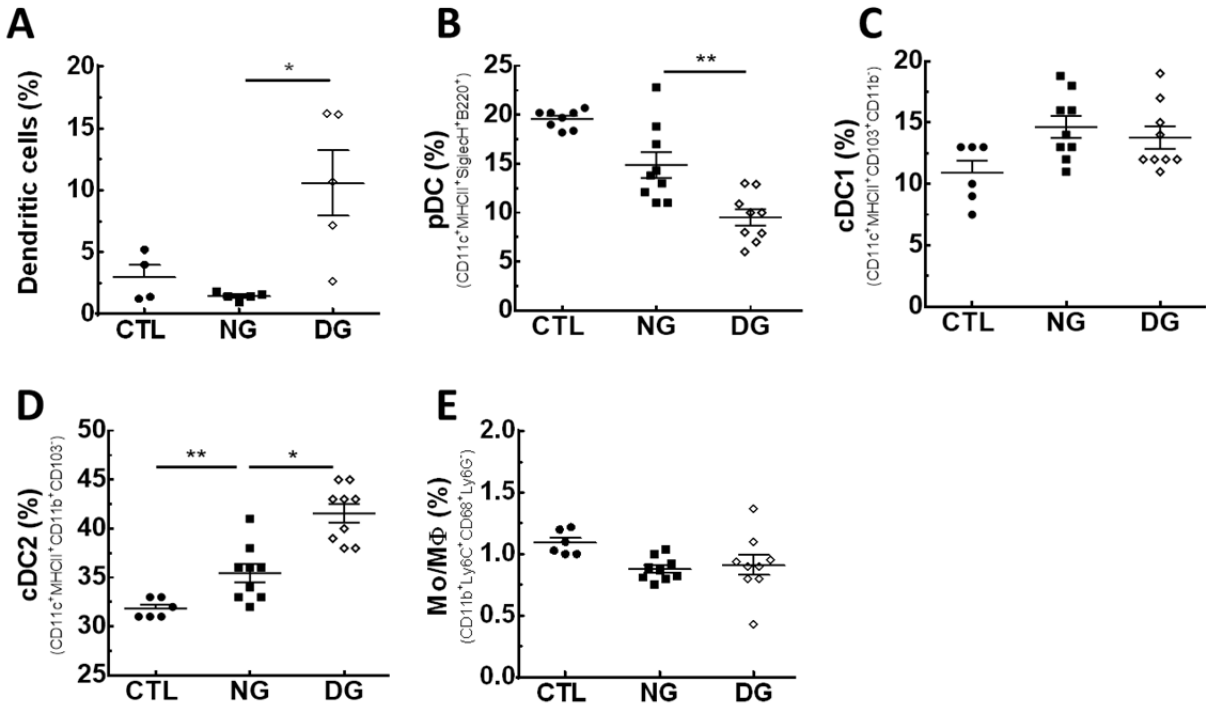

Figure 2. The deamidated form of gliadins induces influx of cDC and defect in pDC. *Frequency of A) DCs, B) pDC, C) cDC1, D) cDC2, and E) monocytes/macrophages in the MLNs after the second sensitisation obtained by flow cytometry from the three groups of mice. Data represent the mean ± SEM (n = 5–10 animals per group); *p < 0.05 and **p < 0.01.*

to control T-cell responses. To examine which sub-population of DCs was modified, we determined frequency of each population of DC. We showed that among DC, pDCs were significantly decreased in DG sensitised mice compared to NG sensitised mice (Fig. 2B). No difference was observed between NG-sensitised mice compared with control mice (Fig. 2B). In contrast, frequency of cDC1 did not differ among the different groups of mice (Fig. 2C). To the opposite, we observed an increase of cDC2 frequency in DG sensitised mice compared with NG sensitised mice (Fig. 2D). Finally, the ratio of monocytes/macrophages frequency did not reveal any difference between sensitised and control mice (Fig. 2E) [11]. To determine whether this increase in cDC2 DCs is associated with lymphocyte differentiation, we measured the Th1/Th2 and the Th17/Treg ratio as they are known to be crucial factors in allergies (Fig. 3). As such, after the first sensitisation, the ratio between $CD3^+CD4^+IL\text{-}4^+$ Th2 cells and $CD3^+CD4^+IFN\gamma^+$ Th1 as well as $CD3^+CD4^+IL\text{-}17^+$ Th17 cells and $CD3^+CD4^+CD25^{high}FoxP3^+$ Treg cells were comparable among the four groups, demonstrating that one sensitisation is not sufficient to induce immune response (data not shown). In contrast, after the second sensitisation, the ratios of Th2/Th1 and Th17/Treg were largely increased in mice sensitised with deamidated gliadins compared to control mice. Moreover, the Th2/Th1 ratio remains unchanged in native gliadin-sensitised mice compared to controls (Fig. 3A). To the opposite, Th17/Treg ratio was significantly increased in DG sensitised mice compared with NG sensitised mice (Fig. 3B). Overall, these results support that the deamidated form of gliadins induces an earlier allergic immune response in mice than the native form of gliadins [11].

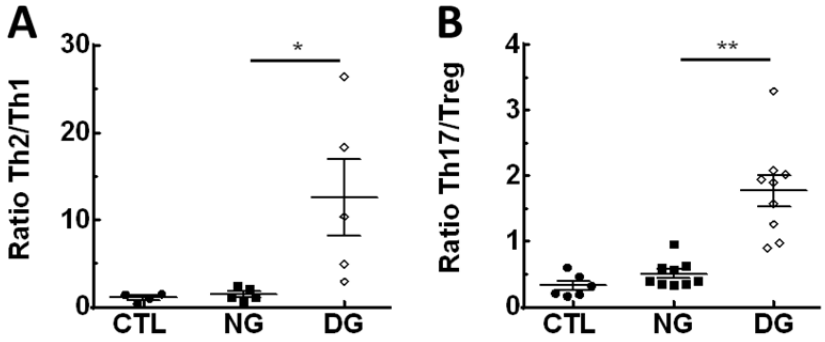

Figure 3. The deamidated form of gliadins induces Th2 polarisation and Th17 inflammation. *A) Th1/Th2 and B) Th17/Treg ratios were measured in MLNs after the second sensitisation and obtained by flow cytometry from the three groups of mice. Th1 ($CD3^+CD4^+IFN\gamma^+$), Th2 ($CD3^+CD4^+IL\text{-}4^+$), Th17 ($CD3^+CD4_+ROR\gamma t^+IL\text{-}17^+$), and Treg ($CD3^+CD4^+CD25^{high}FoxP3^+$) were measured after* in vitro *stimulation. Data represent the mean ± SEM (n =5–10 animals per group); *p < 0.05 and **p < 0.01.*

These results demonstrate the importance of pDC in tolerance establishment by inducing Treg cells, as shown in [19]. Therefore, deamidation of gliadin induces a modification in pDC influx and consequently a stronger sensitisation potential. This is associated with a stronger and a faster presence of cDC2 known for its ability to prime

naive T cells and to generate various effector T (Teff) cell phenotypes because of their prominent expression of MHC class II and costimulatory molecules [20]. Results demonstrate that the stronger sensitizing potential of deamidated gliadins is linked to an earlier attraction of DCs (mainly cDC2) associated with a lack of pDC to mesenteric lymph nodes which in turn induces an earlier Th2/Th17 response. Under normal conditions, the Th1/Th2 cells are in a relatively balanced state in mice. When body dysfunctions such as anaphylaxis occur, they are characterised by a shift in the balance to Th2 cells, thereby resulting in a series of symptoms, including ear swelling, increased gut permeability, and mast cell degranulation. Moreover, a decrease in Treg cells and an inflammation linked to Th17 cells are also observed and participate to inflammation and gut permeability in allergy. Our results were also consistent with the observations of previous studies [21,22], verifying that industrial processing affects the allergenicity of allergens in general.

Conclusions

This study focused on the potential for industrial processes to modify major wheat allergens. The acid hydrolysis inducing deamidation produced greater allergenic potential. In summary, our findings show that industrial processes could modify the allergenic potential of gliadins by worsening its sensitizing capacity. Our data also highlight that deamidation is sufficient for activating key immune pathways necessary for sensitizing mice for immediate hypersensitivity reactions. The data demonstrated the potential risk of partial hydrolysis of food proteins, in which hydrolysed proteins may exhibit increased allergenic potency. Additionally, this experimental model using Balb/c mice may be very useful for further basic and applied studies of the sensitizing potential of various hydrolysed proteins.

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5.5 Kinetics and transcriptomics of gluten-specific T cells after gluten challenge

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Introduction

Coeliac disease (CD) is caused by an inappropriate immune response to gluten proteins found mainly in wheat, rye and barley. Several types of T cells are involved in CD: a) $CD4^+$ T cells that recognise gluten epitopes presented by HLA-DQ2 or -DQ8 and which orchestrate responses of B cells and other T cells [1,2], and b) intraepithelial lymphocytes (IEL) composed of $CD8^+$ αβ T cells and γδ T cells that induce tissue damage [1,3].

Concerning gluten specific $CD4^+$ T cells, Anderson et al. have shown that after a gluten challenge over 3 days in treated CD patients, interferon-γ producing, gluten-specific cells measured by ELISPOT can be found on day 6 after challenge [4]. Gluten specific cells $CD4^+$ T cells can also be detected by HLA-DQ:gluten tetramers on day 6 after challenge [5-7]. Notably, HLA-DQ:gluten tetramer positive cells T cells express gut-homing molecules like β7 integrin and CD103 [5] and the activation marker CD38 [8].

Despite several studies employing HLA-DQ:gluten tetramers [5-7], no kinetic analysis of appearance of gluten-reactive T cells in blood after gluten challenge has been performed. In addition, not much is known about functional aspects of HLA-DQ:gluten tetramer staining T cells besides their cell surface markers which are described above.

Since gluten-specific cells may in future be used for diagnostics and therapy outcome [7,9], a more detailed understanding of their kinetics and cell biology is needed. Hence, we aimed to study the kinetics and specific activation of gluten-specific $CD4^+$ T cells.

Materials and methods

18 CD patients on a gluten-free diet underwent gluten challenge for 3 days with in-house developed, standardised gluten-containing cookies. Blood samples were drawn prior to challenge. In the first study (kinetics study, n=8), cell kinetics in response to gluten challenge were examined, and additional blood samples were drawn on day 1

and 4 – 8. For the second study (transcriptome study, n=10), study participants donated blood samples on day 1 and 6 after the first intake of gluten. 6 participants were challenged with only one cookie (1-day challenge) giving blood samples at baseline and on day 6 after challenge (see figure 1).

Gluten-specific $CD4^+$ T cells were stained with HLA-DQ:gluten tetramers and for *the kinetics study* and *1-day challenge*, their frequency was analysed on flow cytometer as described elsewhere [7]. In *the transcriptome study*, gluten-specific $CD4^+$ T cells underwent subsequent cell sorting and RNA sequencing [9,10].

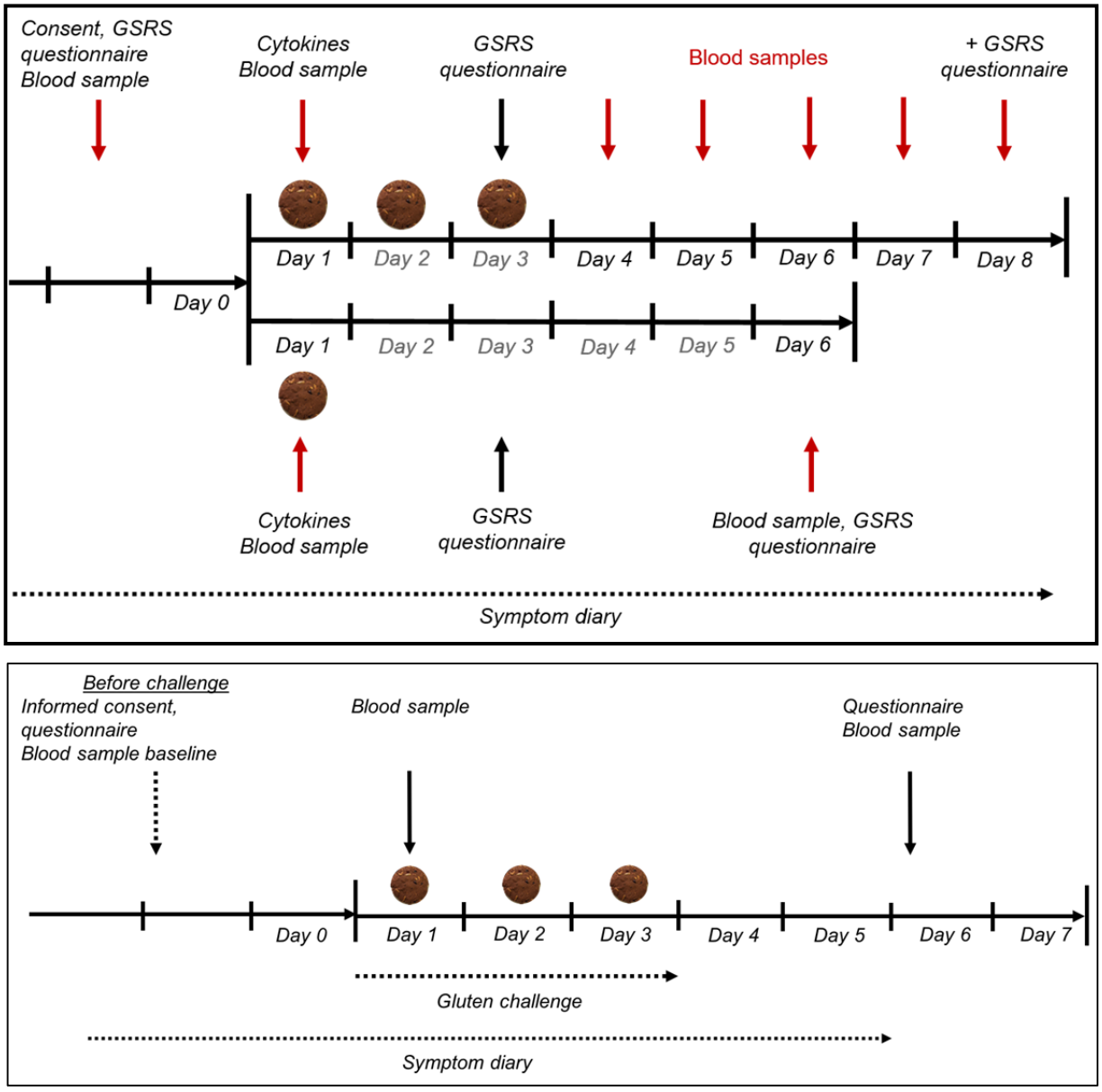

Figure 1. *Timeline for the three gluten-challenge studies: the kinetics study and 1-day challenge (above) and transcriptome study (below)*

The studies are approved by the regional ethical committee of South-East Norway (ref. 2013/1237) and registered at clinicaltrials.gov (NCT NCT02464150).

Results and discussion

All participants gradually increased in tetramer$^+$ cell numbers after gluten challenge over 3 days, plateauing on day 6 to 8 (median 7 vs. 104 cells/10^6 CD4$^+$ cells). 2 participants showed deviating kinetics. 1-day challenge was sufficient to increase tetramer$^+$ cells in all participants, yet resulting in a lower total cell number on day 6 (median 19 cells/10^6 CD4$^+$ cells) (figure 2).

Flow cytometry and transcriptome analysis of gluten-specific gut-homing T cells collected on day 6 after 3-day challenge revealed an upregulation of activation markers (CD28, CD38, CD161, CD82), chemokine receptors (CCR9, CXCR3, CXCR6, CCR6), gut homing markers (CD49, CD103) and IL-21.

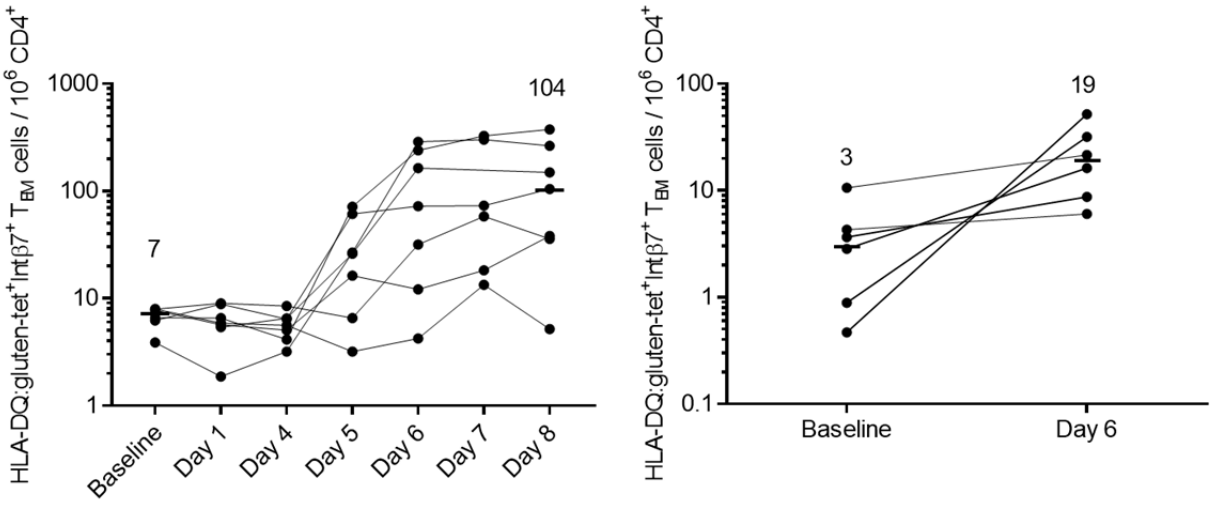

Figure 2. *HLA-DQ:gluten-tet$^+$ T_{EM} cell kinetics (left) and cell numbers after 1-day challenge (right)*

Conclusions

This work provides insights into the kinetics and transcriptomic profile of antigen-specific cells after gluten challenge. Sampling between day 6 and 8 after 3-day gluten challenge is an appropriate time window for collection of gluten-specific T cells. Flow cytometry and transcriptome analysis point to an activated, gut-homing phenotype of gluten-specific T cells.

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6 Symposium Wheat Genomics

6.1 The first reference genome sequence for bread wheat

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Introduction

Wheat is one of the most important crops for human nutrition, but its components are also causative for several human diseases and insensitivities, such as coeliac disease (CD). With the recently published whole genome sequence for bread wheat [1], an important resource is now available to identify and study gene families related to wheat insensitivities and their products in a genome-wide manner. Its complex genetic setup and large genome size of five times the human genome has made the wheat genome impossible to decode for decades. The bread wheat genome consists of three highly similar subgenomes (called the "A", "B" and "D" subgenome, allo-hexaploid) and has a repeat content of more than 80%, making it a grand challenge for short-read sequencing and genome assembly. In 2005, the International Wheat Genome Sequencing Consortium (IWGSC; https://www.wheatgenome.org/) teamed up with the goal to produce a reference genome sequence for bread wheat by the year 2020. Thanks to novel algorithms and bioinformatics strategies, a reference genome sequence along with gene predictions could now be reported even earlier [1]. This new resource established the foundation for in-depth analyses of gene expression and regulation in the bread wheat genome [2] as well as the first genome-wide map of genes and gene families encoding immunoresponsive proteins [3].

CD and wheat allergies such as Baker's asthma and wheat-dependent exercise-induced anaphylaxis (WDEIA) are the most common human diseases associated with wheat grain products (as well as its relatives barley and rye). The majority of proteins involved in these diseases belong to the group of proline-rich storage prolamins [4] and are mostly encoded by gene families. CD is an inflammatory reaction to the components of gluten (gliadins and glutenins) [5], whereas WEIDA and Baker's asthma are allergic reactions to either high-molecular weight glutenins (HMW

[*] these authors contributed equally to this work

glutenins) and omega-gliadins [6] or lipid transfer proteins (LTPs) and alpha-amylase trypsin inhibitors (ATIs) [7], respectively. We therefore focused our research on those gene families. This chapter will describe the progress in establishing high-quality and comprehensive wheat genome resources and discuss the implications and possible benefits for identifying genes and gene families involved in wheat insensitivities as well as for selecting and improving wheat varieties with respect to their potential for human health.

Materials and methods

Making use of the newly established wheat reference genome sequence, potential allergens were identified and mapped in the wheat genome in a first step. For this, members of the Pfam Prolamin clan (CL0482) – *i.e.* Gliadin (PF13016), LTP_2 (PF14368), Hydrophob_seed (PF14547), Tryp_alpha_amyl (PF00234), and Prolamin_like (PF05617) – were used to identify gene members of the prolamin superfamily. HMW glutenins containing the Glutenin_HMW (PF03157) Pfam domain were also included. Finally, the pattern (-C-C-X n -C-X n -C-) was used to identify small cysteine-rich sequences without a proper Pfam domain structure. The AllFam [8] and ProPepper [9] databases were used to identify reference allergens and antigens and to map annotated linear epitopes (i.e. immunoreactive protein sections) associated with CD, WEIDA, and Baker's asthma. Epitope toxicity was inferred from ELISPOT assays used in a previous study [10]. Further details on the identification of potential allergens and epitope mapping can be found in [3].

Second, a phylogenomic analysis was performed comparing bread wheat allergens to nine other Poaceae species (*Aegilops tauschii* – the donor of the D genome, *Triticum urartu* – the donor of the A genome, *Secale cereale*, *Hordeum vulgare* subsp. *vulgare* cv. Morex, *H. vulgare* var. *nudum*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, and *Zea mays*). Protein sequences were filtered and alignments and phylogenetic trees were constructed as described in [1]. These protein sequences and sequences from the UniProt database with PF13016, PF00234, and PF03157 domains were used to further explore epitope prevalence and expansion in bread wheat and related species. See [3] for more details.

Third, gene expression of allergens was evaluated in three different bread wheat lines (Chinese Spring, Norwegian cultivars Bjarne and Berserk) under different temperature conditions. Different grain tissues were extracted (transfer cells, aleurone cells, starchy endosperm) and RNA isolation was performed as described in [11]. Details on RNAseq, mapping, and trimming can be found in [3].

Results and discussion

The availability of a bread wheat reference genome sequence is a milestone in cereal genomics. Its enormous size, high repeat content and hexaploid setup so far severely complicated most genome-wide analyses. The lack of a reference genome sequence

also had consequences for identifying genes and gene families underlying particular traits or associated with certain characteristics such as baking quality. Thanks to novel algorithms and bioinformatics strategies a true reference, chromosome-ordered genome sequence has been reported for bread wheat recently [1], associated with a high-quality gene prediction and annotation. This resource for the first time allows comprehensive, reliable and genome-wide studies of individual genes and gene families. In this context, a first genome-wide map of genes and gene families encoding immunoresponsive proteins was generated [3] and published together with the reference genome sequence.

To guarantee the quality of the annotation of genes encoding immunoresponsive proteins, we performed extensive manual curation of gene models. In particular, Prolamins contain recurring stretches of highly repetitive sequence, complicating automated-only gene prediction severely. We identified 244, 321, and 229 genes encoding immunoresponsive proteins on the A, B, and D subgenome, respectively. In general, the immunoresponsive genes are located in conserved chromosomal areas, usually in the telomeric region of the chromosome (Fig. 1). Thanks to a recent common ancestry and high sequence conservation, this epitope map can also shed light on immunoreative peptides in other cereal species such as barley and rye.

CD-associated epitopes with a high toxicity map mostly in the repetitive region of alpha-, gamma-, omega-gliadins, and low molecular weight glutenins (LMW glutenins) (Fig. 1). Peptides that show a high immunoreactivity are most abundant on the D subgenome – indeed, the proteins showing the strongest immune response are alpha- and omega-gliadins from this subgenome. Phylogenomic analysis revealed that those highly toxic epitopes are only found in the A and D subgenomes, their donors (*T. uratu* and *Ae. tauschii*), and rye. The major epitopes associated with Baker's asthma are ATIs located on chromosomes 3 and 6 of all subgenomes (Fig. 1). Those ATI epitopes were found in all *Triticeae*. WEIDA-associated epitopes can be found in HMW glutenins, gamma- and omega-gliadins – most notably on chromosome 1 in the B subgenome. HMW glutenin-specific epitopes were found in all Triticeae, whereas gamma-gliadin specific epitopes were only found in the bread wheat subgenomes and the A and D donors. The results of the phylogenomic analysis show an emergence of highly toxic epitopes very recently in the Poaceae evolution – and mostly in genes involved in grain and baking quality.

The gene expression analysis showed that low temperature conditions reduced the levels of toxic omega-gliadins. However, low temperature also increased the levels of Baker's asthma- and WEIDA-associated genes (mostly ATIs and LTPs). High temperature, on the other hand, increased the expression levels of CD-associated genes (mostly gliadins and glutenins). Additionally, expression levels differed significantly between genotypes, especially for alpha-gliadins. Identifying effects of temperature fluctuations on grain allergen content will be very helpful for further breeding programs, especially with respect to the anticipated consequences of climate change.

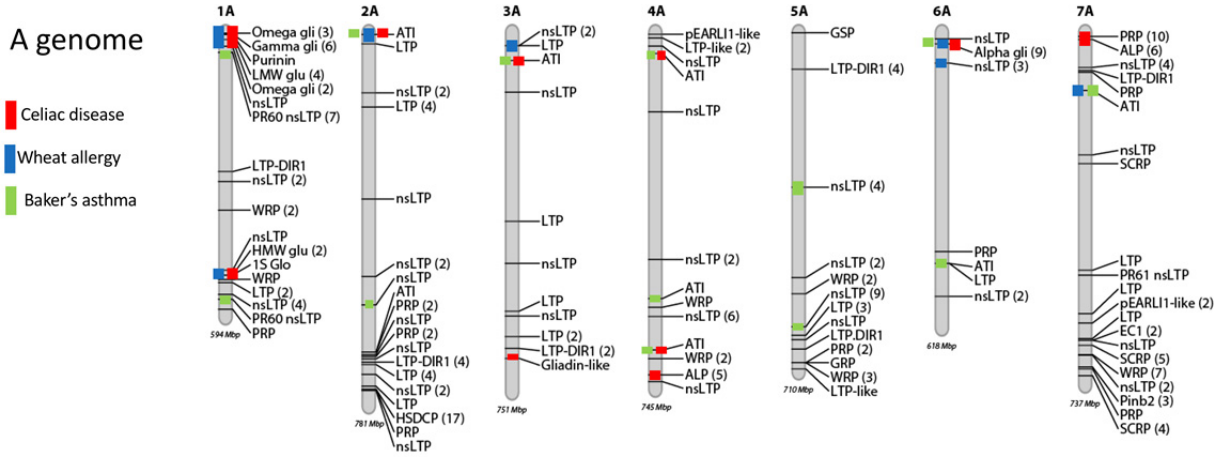

Figure 1. *Distribution of coeliac disease (red), wheat allergy (blue), and Baker's asthma (green) associated genes over the seven A subgenome chromosomes of bread wheat.*

Conclusions

Wheat is one of the major food crops and has many positive nutritional aspects. A small proportion of the human population, however, cannot safely consume wheat grain products. The newly generated reference genome resources for bread wheat allowed us to identify and map genes associated with CD and other wheat associated diseases on the chromosome-scale genome sequence. This map is a first step to enhancing breeding efforts of wheat lines that are saver for human consumption and provide a higher environmental stability. Ongoing wheat pan-genomics and resequencing efforts such as the 10+ wheat project (http://www.10wheatgenomes.com) will greatly contribute towards understanding natural variation and the possible impact of different wheat genotypes on the immunoreactive potential of wheat products. Using a combination of genome sequence data, epitope databases, *in silico* predictions, and gene expression data we work towards a better understanding of the immunoreactive potential of wheat grain end products.

Acknowledgements

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6.2 Gluten genomics in relation to editing coeliac disease epitopes

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Introduction

Gluten are a complex mixture of various proteins in wheat, barley and rye. In wheat it concerns glutenins and gliadins (prolamins). These are storage proteins located in protein bodies in the grain endosperm, encoded by five gene families with up to dozens of members each, clustered in loci on chromosomes 1 and 6. The loci include genes with intact open reading frames, but also genes with premature stop codons, which are considered to be pseudogenes [1]. Starting from one or a few genes in the most recent common ancestor (MRCA) of all Aegilops and wheat species, their number has expanded through tandem gene duplications and gene translocations [2]. The number of genes of the five gene families has now been established for the hexaploid wheat variety Chinese Spring (with the AABBDD genome), of which the whole genome has been sequenced recently using next generation sequencing technologies [3].

Gluten forms a viscoelastic network that is essential for dough making, but gluten also contains several immunogenic epitopes [4] that may cause coeliac disease in genetically predisposed individuals. Coeliac patients have to follow a strict, life-long gluten-free diet, which is difficult to adhere to, especially because wheat flour and isolated (vital) wheat gluten is added to many processed food products for their viscoelastic and binding properties. In addition, the nutritional value, the taste and the overall quality of gluten-free products is often less than their wheat-based equivalents (although these have considerable improved in quality in recent years, along with the steady increase of the gluten-free market), and gluten-free products are more expensive. This means that there is a need to develop coeliac-safe and healthy wheat-based food products. Breeding wheat without the immunogenic epitopes would be a definitive solution [5,6]. The recently developed CRISPR/Cas9 technology for targeted mutagenesis can be a means to establish this.

Here, we review our application of CRISPR/Cas9 to modify gluten epitopes, or to remove entire gluten genes or even complete gluten gene loci, in order to develop coeliac-safe wheat. We start with recent results on the complexity of the gluten genome and the way towards a high-throughput strategy in gluten gene identification (GlutEnSeq) after modification. Further, if such modifications will become successful, replacement of the current assays for *total gluten* detection in gluten-free products (to

confirm a gluten content below the 20 ppm threshold) by an assay for the *absence of immunogenic epitopes* (to confirm safe gluten) is needed. This will be discussed. Finally, we will compare and discuss the results of gene editing approaches on gluten genes by irradiation mutagenesis and by CRISPR/Cas technology from a European legal perspective.

Gluten genomics

To apply CRISPR/Cas9 efficiently, detailed knowledge on the complete set of wheat gluten genes is required. The genome of diploid *Aegilops tauschii* (the donor of the D genome of bread wheat) has been sequenced and assembled [7-9]. Dong et al. [10] studied a region of chromosome 1S (S: short arm) of *Ae. tauschii*, containing 103 genes among which several gamma-, delta- and omega-gliadin and LMW-glutenin genes, with the corresponding (syntenic) region in *Brachypodium* and other *Gramineae* genomes (rice, sorghum). The region in *Ae. tauschii* shared 17 prolamin genes with *Brachypodium* but was 35 times larger compared to the syntenic region in the *Brachypodium*, rice and sorghum genomes, including duplications of prolamin genes and many insertions of retrotransposons. In hexaploid Chinese Spring the Ae. tauschii-derived chromosome 1DS genome region was also much larger compared to the corresponding regions on 1AS and 1BS [11]. The expansion of the genome size during the evolution of the *Triticum/Aegilops* clade has not always led to the same type of expansions, as the 6DS region of Chinese Spring, which includes alpha-gliadins, was only half the size of the syntenic 6BS region [12].

The exact number of gluten genes in hexaploid wheat has long been unclear, with estimations ranging up to 100-150 gene copies for alpha-gliadins alone, including pseudogenes. The genome sequence of Chinese Spring [3] enabled a detailed study of the number of prolamin genes present. Clavijo et al. [13] identified 105 full-length or partial gluten genes (of which 29 alpha-gliadins, 18 gamma-gliadins and 10 omega-gliadins, 6 HMW and 16 LMW) and 13 pseudogenes. This was based on an imperfect annotation, even though it was manually curated, so the numbers may change.

Huo et al. [12] studied the Gli-2 loci, encoding alpha-gliadins, in Chinese Spring in more detail, based on the genome sequence in combination with PacBio reads and BioNano genome maps. They detected 10, 24, and 11 copies of the alpha-gliadins in the A, B, and D genome, respectively. Among these, 26 encoded full-length proteins and 21 (46%) were pseudogenes. Note that other hexaploid wheat varieties and accessions will have different numbers of gluten genes as found in Chinese Spring.

Gluten gene editing with CRISPR/Cas

One can now use CRISPR/Cas9 to remove all gluten genes, which would produce a gluten-free wheat which is interesting for many people who want to eat gluten-free, but it would have an inferior baking quality. An alternative use of gene editing with CRISPR/Cas9 is to precisely modify gliadin genes and strip them of immunogenic

epitopes, to develop wheat with safe gluten. A couple of groups world-wide are applying this technology to wheat to make it coeliac-safe. Sánchez-León et al. [14] in Cordoba, Spain, successfully deleted complete gliadin genes in this way. Alternatively, gliadin genes can be modified to precisely inactivate (by point mutation) or to delete their immunogenic epitopes, which is the strategy in Wageningen, the Netherlands (Jouanin et al., submitted). Next to these European groups, Calyxt (Roseville, Minnesota, US) is working on gene-editing of wheat as well. Ultimately, commercial varieties may be produced based upon a combination of these approaches, with some gluten loci completely deleted and some loci present that only contain coeliac-safe gluten genes, i.e., without epitopes.

To engineer the edits in gliadins, a 20-mer guideRNA (gRNA) plus the Cas9 endonuclease gene is introduced in immature wheat embryos. The gRNA directs the Cas9 nuclease protein to the target site within the gene of interest, where the nuclease generates a double-strand DNA break, three bases upstream of a specific protospacer adjacent motif (PAM). In plants, repair by non-homologous end joining (NHEJ) can result in insertion/deletions (indels) and other mutations creating potentially beneficial phenotypes [15,16], and plants with beneficial mutations are selected from the regenerated plants. Introducing a construct in wheat through genetic transformation was not easy, but Ishida et al. [17] achieved a breakthrough using the US soft white wheat variety, Fielder, employing a supervirulent *Agrobacterium tumefaciens* strain. The introduced construct can be removed in crosses afterwards, simply be selecting construct-free offspring.

An alternative approach for stable transformation is to use transient expression of the CRISPR/Cas9 DNA and the guide RNA. This has been achieved in wheat [18], although it remains to be seen if the efficiency is high enough to edit the multiple gluten targets in one cell; this strategy requires very active promotors.

ddPCR screening

Random mutagenesis, as well as targeted gene editing with CRISPR/Cas9 of gluten gene families will produce offspring with a mosaic of deleted, modified, and unchanged genes (Jouanin et al., submitted). Hence, methods to screen the offspring, as well as the lines from subsequent crosses, are essential. Strategies that aim at producing safe gluten genes without epitopes would require more sophisticated technologies than strategies aiming at wheat with all gluten genes deleted. Exome capture methods have been developed, when combined with next generation (Illumina) sequencing, to provide sensitivity at reasonable costs when hundreds of lines need to be analysed (see below). However, there is still ample need for new and faster methods for the pre-screening phase, when thousands of seeds may need to be screened. Droplet digital PCR (ddPCR) could be one of the techniques used for this. ddPCR enables high-throughput screening of copy number variation (CNV), and Jouanin et al. (in preparation) have applied it successfully for detecting the number of α-gliadins.

SNP markers

Further, marker technology is a helpful tool in identifying and following haplotypes through crosses and might be useful to follow variant alleles. Much progress has been made in this field, thanks to the combination of next generation sequencing technology for cheap (re)sequencing with the technology for high-throughput genotyping using SNP markers. For example, Rimbert et al. [19] performed whole-genome resequencing of eight bread wheat lines. They identified 3.3 million SNPs, 49% on the B-genome, 41% on the A-genome and 10% on the D-genome. The TaBW280K high-throughput genotyping array contains 280,226 of these SNPs, of which 69% can be efficiently scored, half of them showing a diploid-like clustering. This was, for example, used to produce a dense genetic map of Chinese Spring x Renan, comprising 83,721 markers.

Capture sequencing

Although these dense SNP arrays will allow to uniquely tag haplotypes in the wheat germplasm, it will not be possible to differentiate newly made CRISPR/Cas9-edited and coeliac-safe gliadin genes from immunogenic gliadin genes. Resequencing the whole genome of a wheat variety is still a very laborious task. Therefore, we recently developed a DNA capture system, GlutEnSeq, that enables specifically capturing the complete set of gluten genes (including all polymorphisms and modifications) in a variety, an accession, or a progeny plant, after which this set can be sequenced and analysed in detail (Jouanin et al. submitted). The GlutEnSeq system was based on the REnSeq system (for Resistance genes Enrichment and Sequencing [20]) for capturing and sequencing disease resistance genes and alleles in plants.

EU regulation of gene editing

Recently, the European Court of Justice ruled that, according to the text of Directive 2001/18/EC, all mutations artificially induced by gene editing (e.g. by CRISPR/Cas technology) are GM, also if they are identical to mutations that could be induced by random mutagenesis, or already exist elsewhere in nature. This implies that it may require 10-100 M€ to perform all required safety tests for hypo-immunogenic wheat in which gluten genes have been deleted and/or epitopes have been modified. Thus, it is expected that safe or hypoimmunogenic wheat will not be brought on the EU market, in contrast to the situation in the US and in other countries where the safety of the end product of a technology, and not the technology itself, is considered [21].

Mutation breeding

Mutation breeding using gamma-irradiation or EMS, which causes random mutations, is not regulated in the EU. EMS has been used for mutagenesis for many decades, also in wheat. Krasileva et al. [22] analysed a collection of 2735 EMS-mutated bread wheat and durum wheat lines using an 84-Mb exome capture assay followed by sequencing.

They identified more than 6 million mutations in the hexaploid Cadenza background, and more than 4 million in the tetraploid Kronos-derived lines. The lines had on average 2705 (in Kronos) and 5351 (in Cadenza) mutations, equalling 35-40 mutations per kb. Even at this high number of mutations, it would be very difficult to identify lines in which many gluten genes would be mutated simultaneously, in the desired way towards coeliac-safety. Combining the mutations from various lines by crossing may be an option, followed by backcrossing to Cadenza or Kronos to remove most of the other mutations, but identification of the mutants at the DNA sequence level is still required.

The statistics become more attractive when one does not need to mutate all gluten genes separately, but when a gene that influences their expression could be targeted. Moehs et al. [23] developed a decreased-gluten wheat using EMS-mutated alleles of the wheat homolog of lys3a in barley. For this they screened >10,000 M2 plants and found as many as 350 mutations in the three wheat homoeologs of lys3a, a prolamin-box binding factor that that leads to a higher content of free lysine in barley as a consequence of lower prolamin synthesis. By combining three mutations they already achieved 50-60% reduction in gliadin and LMW-glutenin accumulation. The authors work for Arcadia Biosciences, California, USA, who develop and market varieties with health benefits (lower gluten or slow-degrading starch). The approach is based upon random mutagenesis methods in combination with high-throughput screening, and then combining the mutations by crossing.

Wheat with coeliac-safe gluten or without gluten

Gliadin and glutenin genes without immunogenic epitopes do exist, but are rare and always accompanied by many gluten genes with one or more epitopes. Wheat varieties that are safe for coeliacs do not exist naturally and cannot be produced by conventional breeding alone [6], because there are too many genes involved (too few of which have coeliac-safe alleles) which are clustered on different chromosomes in each of the three genomes in such a way that recombination is not effective to combine only the safe alleles. Deleting blocks of genes with traditional mutagenesis leads to the loss of more and different genes, which affects the fitness of the resulting plants. Deletion lines, e.g. in Chinese Spring, in which parts of chromosome arms with gluten loci have been eliminated by mutagenesis, are useful research tools, but usually not considered suitable material for a breeding program.

An exception to this rule is the Ultra Low Gluten variety produced in diploid barley. For this, gamma-irradiated accession Risø 56 (lacking B-hordeins), Risø 1508 (lacking C-hordeins) and R118 (an Ethiopian derived line which lacks D-hordeins) have been intercrossed to produce ULG 2.0, which has a hordein (gluten) content below 5 ppm, thus allowing it to be classified as ‘gluten-free’ [24]. Subsequent crossing and selection with commercial varieties resulted in a variety with good malting and brewing characteristics, marketed as ‘Kebari’ and currently used for the production of gluten-free beer in Germany. Interestingly, in several countries the products made with

this variety cannot legally be marketed as gluten-free, solely because of the fact that it contains barley.

Conclusion

Comparing the different strategies for mutagenesis, the targeted approach using the CRISPR/Cas technology is promising and involves a relatively simple and directed detection protocol for adequate analysis of the modified wheat lines: ddPCR followed by capture sequencing. Further, towards the end of the development of gene-edited coeliac-safe wheat lines, proteomics methods are needed to precisely characterise the protein composition of the grains to fully confirm the desired gene editing.

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7 Statements on current developments concerning gluten analysis, clinical and legal aspects

7.1 Gluten-free diet & irritable bowel syndrome

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Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterised by abdominal pain, discomfort and alteration in bowel habits. IBS is also responsible for psychological disorders like anxiety, depression and a general diminishing in quality of life [1]. This disorder affects approximately 4-20% of western population.

IBS is connected to coeliac disease (CD) by presence of similar symptoms (diarrhoea, nausea, abdominal pain), which causes, in some instances, CD to be misdiagnosed for IBS. Non-celiac gluten sensitivity (NCGS) is characterised by intestinal and extra-intestinal symptoms related to the ingestion of gluten/wheat - containing food, in subjects that are not affected by either CD or wheat allergy [2].

NCGS was described recently, after some cases of patients with symptoms very similar to CD and IBS, but without abnormalities at intestinal cells, and whose symptoms improved after an adherence to a gluten-free diet (GDF).

Fig. 1 shows the overlap between IBS, NCGS and CD symptoms. Symptoms are shared between CD, IBS and NCGS, and all three conditions seem to have a benefit from a gluten-free diet.

Causal agents for IBS are still under study and there are many hypothesis. Even if sugar components are described as the most possible cause, Aziz et al. ^$ et al [1] suggested also other components in wheat as responsible for IBS. Beside Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols (FODMAPs), which are short-chain carbohydrates causing gas production and osmotic diarrhoea, there are: i) wheat germ agglutinin, which are known to stimulate the pro-inflammatory cytokines; ii) amylase trypsin inhibitors (ATIs), which can stimulate the innate immune response, activate the TLR4, and increase T-cell adaptive response and iii) gluten that stimulates the innate immune response, inducing the proliferation of monocytes, the expression of TLRs and INF-γ and altering intestinal permeability [3].

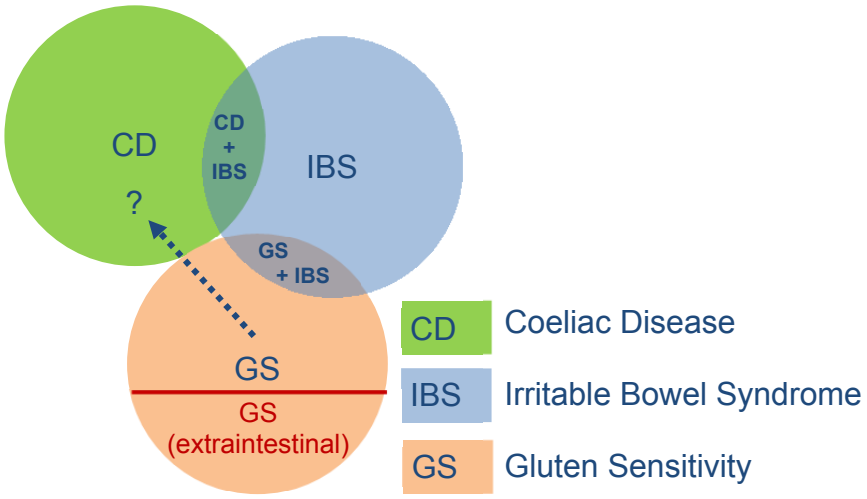


Figure 1. *Overlapping between IBS, NCGS and CD [4].*

FODMAPs and IBS

Therapy for IBS has not been discovered, while it is known that some kinds of foods trigger IBS symptoms, researchers are still looking for an effective way to treat IBS. Diet, in some cases, can be helpful; in particular, a low FODMAPs diet can reduce symptoms. Already in 2006 research examined the role of FODMAPs in IBS. One study showed a symptomatic improvement in 74% on a low-fructose/fructan diet [5].

Since then, several randomised control trials have demonstrated the efficacy of the low FODMAPs diet and probable mechanisms [6].

Halmos et al. [7] have studied the effect of a FODMAPs-free diet in people with IBS, in comparison with a high FODMAPs diet. Low FODMAPs diet was consumed for 21 days by 30 patients with IBS and 8 healthy individuals, followed by consumption of a typical Australian diet, with a daily content of 4,4g oligosaccharides and 2,6g polyols, for other 21 days. Depending on the type of diet, differences in symptoms were reported. In IBS patients treated with low FODMAPs diet the results (Fig. 2) indicated a clear decrease in pain and disturbs linked to the IBS, including low bloating (A) and abdominal pain (B), a reduced faecal frequency in case of IBS-D (with diarrhoea) in D graph, less dissatisfaction of stool consistency in IBS-C (with constipation) in E.

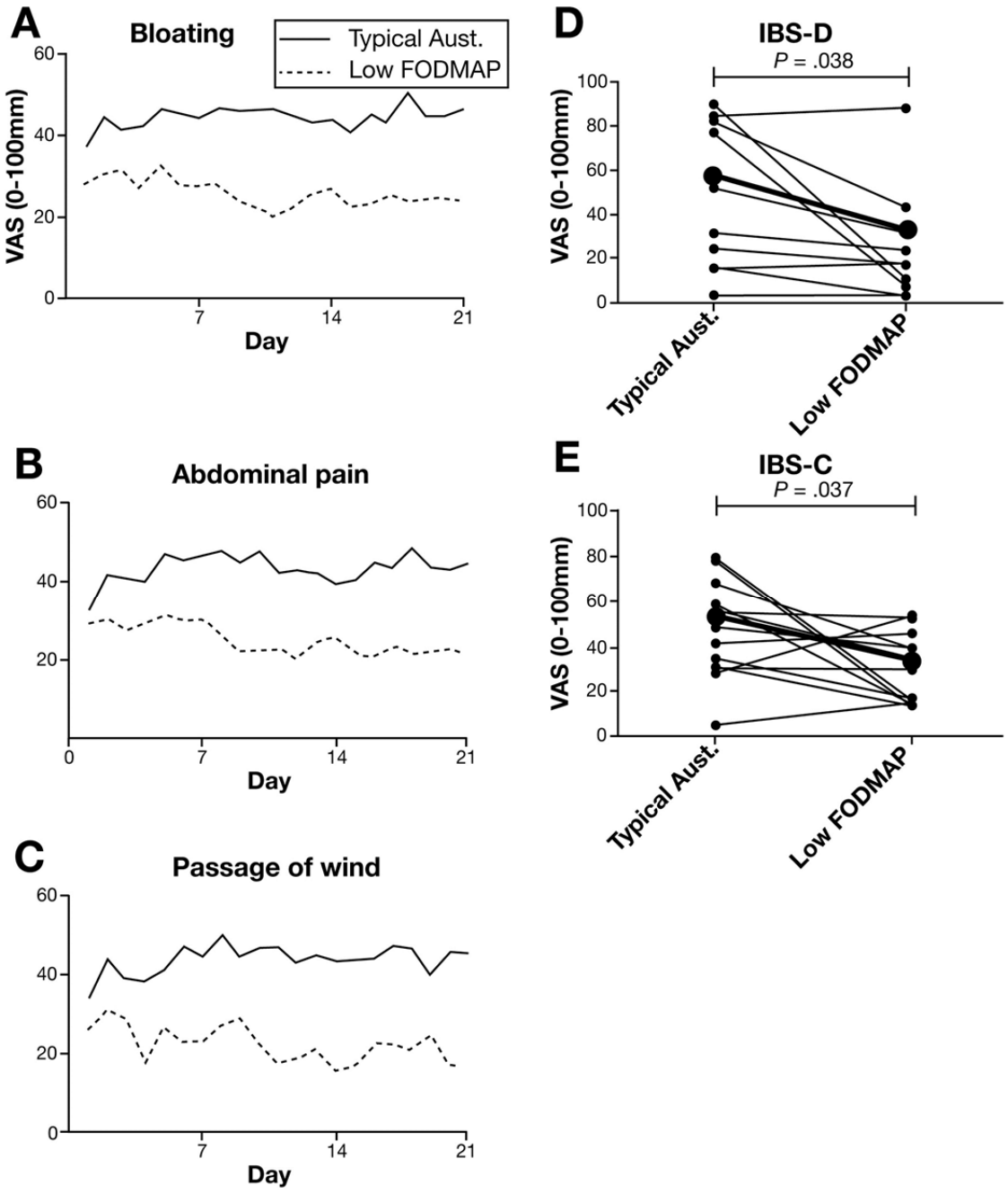
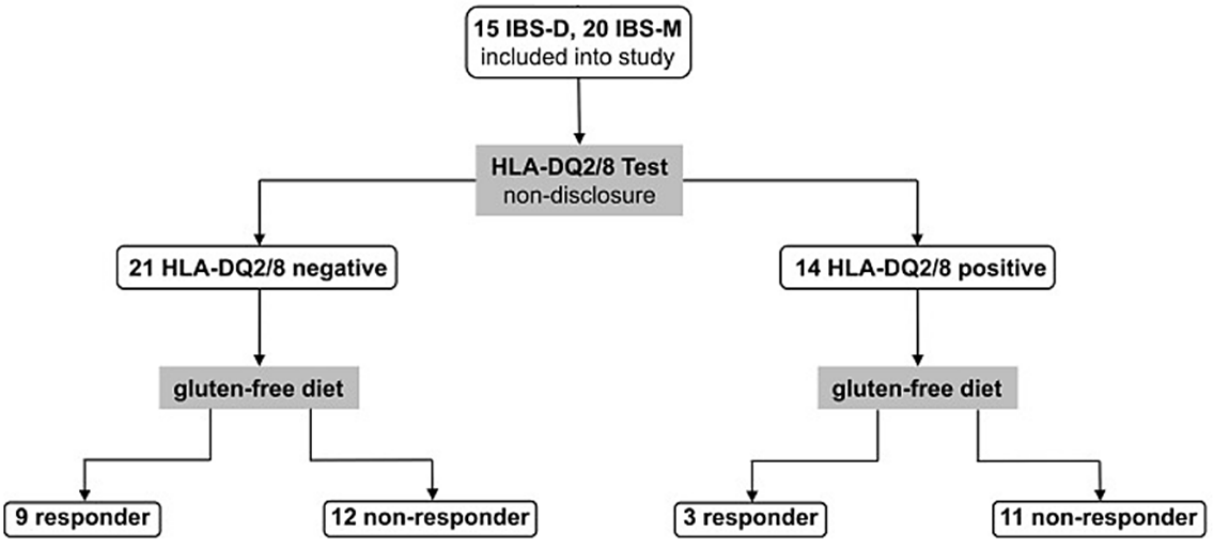


Figure 2. *Mean of symptoms of IBS during a typical Australian diet and low FODMAPs diet according to Halmos et al. (2014) [7].*

Relation between Gluten and IBS

Research recently has focused also on the role of a gluten-free diet in IBS. The benefit of a gluten-free diet in patients especially with IBS- D (and also IBS –M) has been shown in different studies.

A clinical study demonstrated that GFD can improve symptoms in IBS patients [8]. A GFD was followed by a group of IBS patients with different type of symptoms (diarrhoea-dominant IBS-D and mixed type IBS-M). Among these, 34% were responder to GFD, feeling a symptoms relief (Fig. 3).



	HLA-DQ2/8 positive	HLA-DQ2/8 negative	IBS-D	IBS-M
Responder	3 (21 %)	9 (43 %)	5 (33 %)	7 (35 %9
Non-responder	11 (79 %)	12 (57 %)	19 (67 %)	13 (65 %)

Figure 3. *Clinical study on 35 IBS patients supplied with GFD.*

In a prospective study of 41 patients with IBS-D all participants were placed on a six week gluten-free diet following advice by a dietitian, at six-week follow up. A GFD had significantly reduced "IBS Symptom Severity Scores" in 71% of the cohort and the mean total IBS symptom Severity Scores decreased from 286 to 131 points [9].

Even if the trigger of IBS is still not clear, in many studies it is reported that the GFD brings to symptoms relief. This is probably due to low concentration of FODMAPs, agglutinins, ATIs and gluten in the gluten-free foods.

Conclusions

IBS is a disorder connected with GS and CD. Its pathophysiology mechanism is not clear and causal agents are still not identified. So far, GFD is a possible therapy, not only for CD, but also for some IBS and NCGS patients. Further studies about IBS, diet, and role of wheat in IBS are necessary for a better understanding of this syndrome, as well as for the identification of all the critical elements that can trigger symptoms and for the design of dedicated gluten-free food solutions.

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7.2 On deamidation and hydrolysis of wheat gluten

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Introduction

Non enzymatic deamidation is a post-modification event that has been observed and characterised in a wide variety of proteins playing a role in several diseases, and is believed to relate to aging effects acting as a molecular clock [1,2]. It has been shown to regulate some time dependent biological processes for the determination of the age of e.g. bones [3]. There are many reports on deamidation under physiological conditions in proteins of biological significance. Deamidation occurs *in vitro* and *in vivo*, and the rates of deamidation depend on multiple factors, including the primary sequences and three-dimensional structure, pH, temperature, and buffer salts in the solutions [4].

Under mildly alkaline conditions, asparagine deamidation starts with the nucleophilic attack of the peptide nitrogen on the side chain carbonyl of the following peptide group leading to the formation of an asymmetric five-membered succinimide ring intermediate that is quickly hydrolysed into D,L-aspartate and D,L-isoaspartate in a ratio of approximately 1:3 [5-8]. The latter results in a β-shift in the primary sequence. Deamidation of glutamine is as much as ten times slower because it is thermo-dynamically less favourable to form a six-membered glutarimide ring [9-11]. As a free amino acid, or as the N-terminal residue of a peptide or protein, glutamine deamidates readily to form pyroglutamic acid (5-oxoproline). The reaction proceeds via nucleophilic attack of the α-amino group on the side-chain amide to form a γ-lactam with the elimination of ammonia from the side-chain. At low pH (< 2), direct hydrolysis of the side chain amide generates aspartate as the sole product without the formation of a cyclic imide intermediate. Deamidation causes an increase in the mass of 0.984 Da due to replacement of an amide into a carboxylic group.

The purpose of this contribution is to highlight protein chemical insights on deamidation and hydrolysis of wheat gluten and oilseeds proteins showing that chemical deamidation is not at random process but determined by the amino acid composition and sequence of the protein source. Deamidation is one of the major degradation process of proteins and peptides. Caution should be taken when storing and preparing samples for analysis.

Deamidation in food proteins

Major cereal and oilseed proteins contain non-essential amino acids between 55 to 69 % with cereal flours covering the higher end (57-69 %) [12]. Oilseed proteins are

containing higher levels of arginine and lysine. Glutamine and asparagine are making up 23-43 % of the total amount of amino acids. Table 1 is summarizing some relevant data on glutamine and asparagine levels of different protein sources. The ratio between glutamine to asparagine in cereals is quite high (for wheat derived products 7 or higher even higher than 10 for wheat gluten) compared to the ratio found in other protein sources (below 3). Another feature is the level of amidation : in cereal products levels between 80 to 96 % are found, while in oilseed proteins the level of amidation is about 50 to 60 %.

Table 1. *Glutamine and aspartic acid parameters from major cereals and oilseeds crops*

	Amino acid (% on protein base), ratio and degree of amidation			
Protein source	**Glx**	**Asx**	**Ratio Glx/Asx**	**Degree of amidation[f]**
Wheat flour[a]	31.1 %	4.2 %	7.4	88 %
Barley flour[a]	24.8 %	4.9 %	5.1	91 %
Oat flour[a]	19.5 %	8.1 %	2.4	70 %
Wheat gluten[b]	38.0 %	2.6 %	14.6	96 %
Gliadin[b]	41.1 %	2.3 %	17.9	89 %
Glutenin[b]	36.8 %	2.8 %	13.1	80 %
Soybean meal[c]	19.7 %	11.6 %	1.7	51 %
Sunflower isolate[d]	26.2 %	10.1 %	2.6	N.A.
Cottonseed[c]	22.3 %	10.4 %	2.1	58 %
Casein[c]	20.2 %	6.4 %	3.2	51 %
Lysozyme[e]	4.9 %	19.9 %	0.24	7.6 %

[a] Ref. [12] [b] Ref. [13-14] [c] Ref. [15] [d] Ref. [16] [e] Ref. [17] [f] Calculated from ammonia derived from glutamine and asparagine versus sum of glutamic and aspartic acid. N.A., not analysed.

The amino acid composition of wheat gluten is characterised by a high glutamic acid content, a relatively high proline content, a low amount of basic amino acids (lysine, arginine, histidine), a high degree of amidation nearly equivalent to the aspartic and glutamic acid content and a remarkable cysteine content. Aspartic acid content in wheat gluten is lower compared to the level in wheat flour. The mean percentage of (glutamic + aspartic) acids in the amide form is 82 for glutenin and 92 for gliadin [18,19]. More recent determinations on amino acid composition on gluten, gliadin and glutenin [20] revealed that the ratio of glutamine to asparagine was between 10 to 11**.** A high degree of amidation increase the hydrogen bonds interactions and decrease solubility. Lásztity [21] showed that the relation between the degree of amidation and the relaxation time of gluten showed an optimum at 88 % of amidation. Deamidation introduces a negative charge at the reaction site and can also lead to structural isomerisation especially around aspartyl residues containing amino acids with low steric hindrance. The extent and rate of deamidation is depending on the ratio of

glutamine and asparagine and the degree of amidation in the native protein. pH is also very important, not only to the extent of deamidation but also to the mechanism of the reaction. Zhang *et al.* [22] compared the kinetics of deamidation of soy protein isolate with that of lysozyme. The deamidation rate of lysozyme was higher compared to that of soy protein isolate which was explained by a much higher amount of asparagine compared to glutamine in lysozyme. On either side of aspartic acid peptide bonds may be cleaved at a rate at least 100 times greater than other bonds under carefully controlled conditions in dilute acid. Free aspartic acid was observed 30 minutes after the start of acidic deamidation of soy protein isolate which increased over time [23]. Deamidation with food proteins at large scale is mainly conducted by using acids [24].

Deamidation and hydrolysis of wheat gluten

A number of authors [25-28] have established that deamidation and hydrolysis of wheat gluten go hand in hand. Finley [27] prepared suspensions of 5-10 % gluten with hydrochloric acid of different strengths between 0.025 to 1 N HCl and reacted them during 30 minutes at 50, 75 and 95 °C. Degree of hydrolysis and deamidation were monitored. Peptide hydrolysis requiring much harsher reaction conditions (6 N HCl at 110 °C during 24 hours) as for amino acid analysis and can be viewed as hydrolysis of a secondary amide, while complete deamidation only requiring treatment with 2 N HCl at 110 °C during two hours. The sum of deamidation (= hydrolysis of primary amide) and hydrolysis are expressed as hydrolysis of amide groups and is represented in Fig. 1.

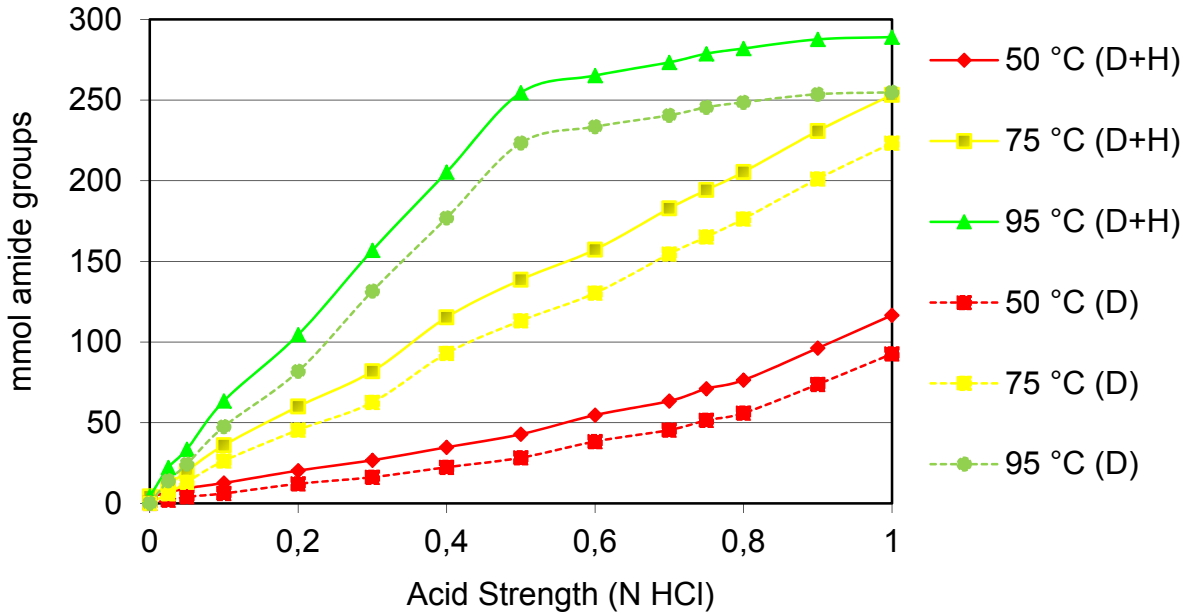

***Figure 1**. The effect of acid strength on the sum of deamidation (D) and hydrolysis (H) of gluten suspensions at 50, 75 and 95 °C (data replotted from Finley, 1975 [27]).*

As with most reactions, higher temperatures will increase the rate of the deamidation reaction. Deamidation is for each treatment the main contributor in amide hydrolysis compared to peptide hydrolysis although at 95 °C the contribution is remarkably lower.

The contribution of hydrolysis is increasing only slightly as deamidation is progressing to a much larger extent. In Fig 2 the Finley data has been replotted for each data point and revealing that at each temperature the increase of deamidation and hydrolysis follows the same pattern. Up to 25 % of deamidation the % DH (degree of hydrolysis) is limited to 2.5-3 % while by levels of deamidation above 25 %, the hydrolysis goes to a pace with is only one fourth of the initial level. This is explained by changes in the higher-order structure in the protein [29]. A similar behaviour is observed by deamidation of soy protein isolate although at 36 % deamidation a 4.5 % DH is already obtained. This might be explained by the presence of more aspartic acid residues in the soy protein at start which are most likely less amidated compared to wheat gluten.

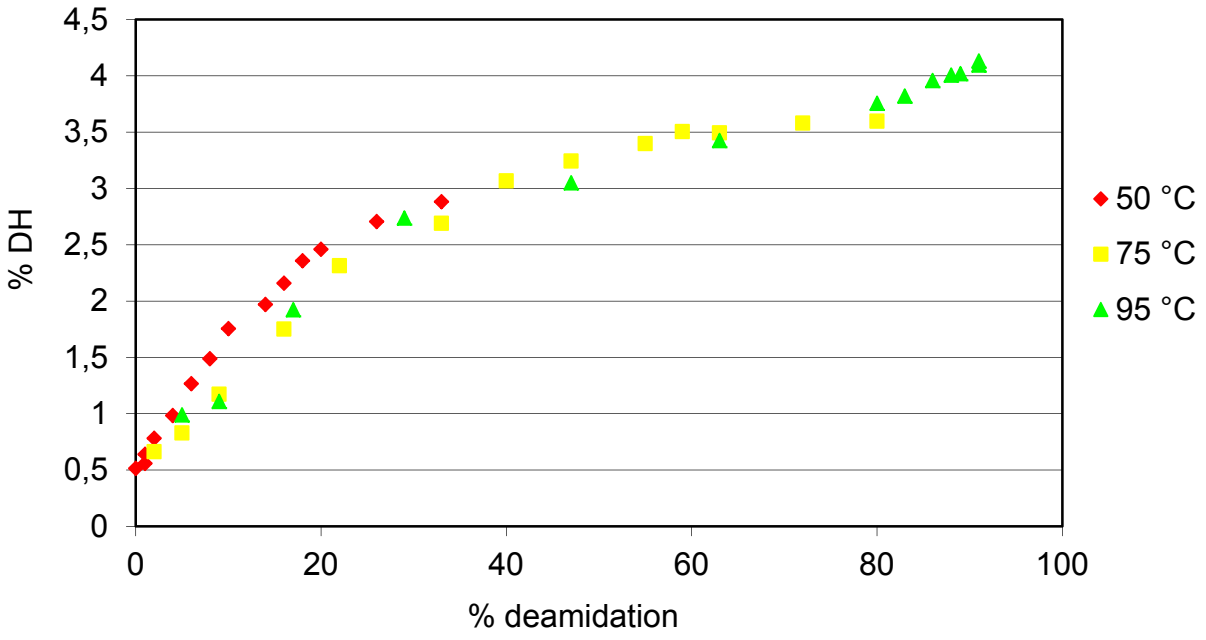

Figure 2. *Recalculation and representation of the data from Fig. 1 and [27]. For each data point the degree of hydrolysis of the peptide bond is expressed as a function of the degree of deamidation.*

Effect of deamidation on protein properties

Deamidation also was reported to increase exposed hydrophobicity of gluten induced by a conformational change and, subsequently, to increase surface activity [29]. The functional properties as emulsifying capacity and stability were increased greatly. A number of studies investigated the molecular mechanism for protein structural destabilisation upon deamidation. For example, the deamidation treatment has direct

implications for charge density and, in turn, affects electrostatic interactions the protein may undergo by interacting with water or upon self-assembly [9,27,30]. This role of electrostatics to deamidation induced disruption of protein structure was supported by further observations on wheat gluten: both acetic acid and HCl induced deamidation had substantial consequences for the secondary structure of wheat gluten. It was thus postulated that strong deamidation induced protein unfolding as a result of electrostatic repulsion [31]. Acetic acid induced deamidation of wheat gluten was further found to inhibit SDS-stable aggregate formation whilst largely retaining its ability to form disulphide bonds [31]. The rate of the deamidation reaction has been found to depend on primary sequence and pH under which the reaction takes place, but was independent of ionic strength for model peptides [4,7,8,32], soy protein and egg white lysozyme [22].

As glutamic and aspartic acid residues are the main constituents of the β-turn form, the increase of β-turn might be due to the fact that more glutamic and aspartic acid residues were exposed as the degree of deamidation increased. As a result of increased β-turn in modified samples, the charge density and electrostatic repulsion of protein consequently became stronger after deamidation with HCl, facilitating unfolding of the protein conformation and protein−water interactions and improving the susceptibility of wheat gluten to enzymatic hydrolysis [33,34]. The increase of charge and decrease of β-sheet make the proteins more flexible after deamidation, especially N-terminal domain which was rich in glutamine residues. In comparison, the slight increase of α–helix which appeared mostly in C-terminal domain [35] means that C-terminal domain is more resistant to changes induced by deamidation. Qiu et al. [34] reported that wheat gliadin deamidated by HCl exhibited an increase of β-sheet while a decrease of the α-helix was observed.

Deamidation using four different organic acids providing a similar degree of deamidation with concomitant hydrolysis at the same levels as previously published. Wang *et al*. [36] showed that the solubility and emulsifying property of deamidated wheat glutens were significantly improved, and the essential amino acid contents were enhanced. Circular dichroism and Fourier transform infrared spectroscopy showed there was a decrease in β-sheet and a slight increase in random coil of deamidated wheat glutens. Deamidation of wheat gluten by succinic acid [33] was found to be more efficient than that with citric acid, although wheat gluten treated with succinic acid exhibited less improvement in the foaming capacity and stability and experienced inhibition in the emulsification activity compared with the gluten treated with citric acid. Wheat gluten deamidated with citric acid exhibited more flexible protein molecules, greater changes in the tertiary and secondary structures. Moreover, succinic acid deamidation-induced modification resulted in little change in molecular weight and secondary structure of the protein. Thus, succinic acid could facilitate unfolding protein conformation. Moisture-heating (121 °C during 10 min) in the deamidation pre-treatment decreased the susceptibility of wheat gluten to enzymatic hydrolysis and the peptide factions of ≤ 3000 Da in the hydrolysates due to the formation of larger molecule weight aggregates.

Conclusions

Industrially deamidated wheat glutens are manufactured using acids providing products with a degree of deamidation between 15 to 60 % [24]. However, although the same degree of deamidation (60 %) and hydrolysis was obtained using different organic acids, differences in the release of free amino acids was evidenced [37] following a complementary enzymatic hydrolysis during 48 hours using pancreatin. Most likely due to a different secondary and tertiary structure in deamidated wheat gluten brought by deploying different organic acids leading to the formation of carboxylic groups and reduction of hydrogen bonds in a different high order structure.

Based on the faster deamidation at neutral and alkaline deamidation it is important to take into account non enzymatic deamidation that easily occurs during sample preparation using pancreatin at pH of 9. The impact on proteomic sample preparation has not been systematically investigated. Mild acidic conditions eliminating deamidation during proteolysis could improve conclusions on deamidated wheat gluten when peptides are investigated. Recently, similar recommendations were published for investigating the complexity of allergic diseases that approaches combining accurate product characterisation of deamidated wheat gluten, *in vitro* and animal models studies need to be considered [24].

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8 Perspectives and action plan of the PWG

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The Prolamin Working Group executive meeting and joint discussion held on 28 September 2018, led to the decisions and statements outlined below.

Action plan

I. Analytical

- Efforts to find an alternative distributor for PWG-gliadin have finally been successful. From 1st January, 2019, the material is available from the Arbeitsgemeinschaft Getreidedeforschung e.V. (Association of Cereal Research), Detmold, Germany.
- Alternative reference material: The MoniQA initiative has extensively characterised wheat cultivars from around the globe. A flour of a blend of five wheat cultivars will shortly be available as a reference material for purchase.
- However, the PWG considers flour not as a suitable reference material and supports a protein sample as reference material. Further action is planned in this field.

II. Clinical

- To improve the visibility of the group, Open Access publications with scientific opinions of the PWG on current issues of cereals and gluten will be made.
- The PWG considers being a working group under the umbrella of the International Society For The Study Of Celiac Disease

III. Members, Policy

- Carmen Gianfrani is a new member of the group.
- Martin Stern, Thomas Mothes and Rudolf Valenta left the group.
- New group members have to be identified because some members will retire in the next years.
- This printed, citable book (print run: 250 copies with ISBN number) was made possible by funding of Dr. SCHÄR GmbH/Srl, (Burgstall, BZ, Italy). It will be distributed among leaders of opinion in gluten analysis and clinical medicine. An electronic version can be downloaded free of charge from the PWG website (http://www.wgpat.com).

Next meeting: 2019

We are very pleased to announce the venue for our meeting in 2019:

Urbino, Italy

Host:

Prof. Dr. Carlo Catassi
Università Politecnica delle Marche
Ancona, Italy
E-mail: c.catassi@staff.univpm.it

Time: 10 - 12 October 2019

Focus of the meeting:

- Research on amylase-trypsin inhibitors (ATI)

The meeting will be limited to 55 participants and attendance is by invitation only. Invitations will be sent by April 2019. Registration deadline will be June 15, 2019.

Very special thanks to the hosts for this kind invitation!

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