Proceedings of the 24th Meeting

WORKING GROUP on PROLAMIN ANALYSIS and TOXICITY

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University of Tübingen

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Impressum

Proceedings of the 24th Meeting

WORKING GROUP
on PROLAMIN ANALYSIS and TOXICITY

30 September - 2 October, 2010
Ancona, Italy

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Introduction

The 24th meeting of the Working Group on Prolamin Analysis and Toxicity (PWG) took place at the University of Ancona, Facoltà di Economia, Ancona, Italy, from September 30 to October 2, 2010. Our hosts, Professor Carlo Catassi from Università Politecnica delle Marche and AiC - Associazione Italiana Celiachia, Genova, Italy, as well as Elisa Giordano from forum service, Genova, Italy, welcomed the group, the invited speakers and participants from industry (cereal starch producers, producers of gluten-free food, manufacturers of kits for gluten analysis) as well as representatives from international and national coeliac societies who attended the meeting.

The Prolamin Group meeting aimed at continuing the analytical and clinical discussion initiated by Codex Alimentarius concerning gluten analysis and the control of food for special dietary use for persons intolerant to gluten. A special symposium was devoted to gluten sensitivity beyond coeliac disease and to novel therapies.

I am grateful to all participants for their active contributions, in particular to Carlo Catassi, AiC and forum service for their kind hospitality and efficient organisation of the meeting. I express my gratitude towards all friends, colleagues and sponsors for their inspiration and continued support.

January 2011, Tübingen Martin Stern

Proceedings of the 24th Meeting Working Group on Prolamin Analysis and Toxicity (PWG)

September 30 - October 2, 2010, Ancona, Italy

Executive Summary

The meeting focused on gluten analysis and the control of food for special dietary use for persons intolerant to gluten. Beyond this the spectrum of gluten sensitivity was extended and novel alternative therapies were discussed.

Analytical reports

Seven reports focused on ELISA methods for gluten analysis including the detection of toxic gluten fragments. New test kits were described to be investigated further by ring trials.

Clinical reports

Five clinical reports focused on oats, quinoa and on special findings in pathophysiology and immunology of coeliac disease.

In a special symposium, gluten sensitivity was taken beyond the limits of coeliac disease to include wheat allergy and a full spectrum of non-coeliac clinical forms of gluten sensitivity, at gastrointestinal, skin and neurological level. Studies into enzymatic fermentation including bacterial and barley peptidases and into microbial transamidation of gluten indicated alternative ways of therapy which might be used as an adjunct to the gluten-free diet in the future.

A statement from the Association of European Coeliac Societies (AOECS) was given. The meeting led to a new understanding of the importance of gluten analysis.

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Program

THURSDAY, 30 September, 2010

20:00 Arrival of Prolamin Group and all participants
Informal get-together
Location:
Ristorante della Rose, Marina di Montemarciano

FRIDAY, 1 October, 2010

09:00 THE PROLAMIN WORKING GROUP RESEARCH REPORTS / ANALYTICAL

- Analytical reports (Chirdo, Ciclitira, van Eckert, Janssen, Köhler, Koning, Mothes; guests)

13:00 Lunch

14:00 THE PROLAMIN WORKING GROUP RESEARCH REPORTS / CLINICAL

- Clinical reports (Catassi, Chirdo, Ciclitira, Feighery, Kaukinen, Koning, Mothes, Stern, Troncone; guests)

16:00 Coffee break

16:30 THE PROLAMIN WORKING GROUP EXECUTIVE MEETING (members only)

20:00 Joint dinner of all participants and Concert
Location: Teatro delle Muse, Ancona

SATURDAY, 2 October, 2010

09:00 Gluten sensitivity: the new kid on the block of gluten spectrum disorders (Prof. Alessio Fasano, M.D., Baltimore, MD, USA)

SYMPOSIUM **Alternative and novel therapies** (Chair: Prof. Carlo Catassi, Ancona, Italy)	
10:00	Presumptive safety for coeliac patients of wheat-baked goods rendered gluten-free during sourdough fermentation (Dr. Marco Gobbetti, Bari, Italy)
10:40	Transamidation of wheat: an enzyme strategy to detoxify gluten (Dr. Mauro Rossi, Avellino, Italy)
11:20	Peptidases for degradation of gluten and possible use in dietary therapy (Prof. Dr. Peter Köhler, Garching, Germany)

10:15 Discussion of current developments concerning gluten analysis and clinical developments

Statements by participating organisations (AOECS, ISDI), representatives from industry and guests

- Outline: Action Plan PWG 2011

13:00 Lunch and Farewell

Afternoon

- Extra time for informal meetings and additional PWG executive meeting concerning Action plan

SUNDAY, 3 October, 2010

Departure of the PWG

I. Analytical research reports

Comparison of different protein references and ELISA kits for the detection of gluten in foods

Theresa Schwalb, Herbert Wieser, Peter Koehler

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Introduction

Coeliac Disease (CD) is one of the most common food intolerances. It comes along with serious damage of the mucosa in the small intestine and is caused by the storage proteins – termed 'gluten' – of wheat, rye, barley and, possibly, oats (for a summary cf. [1]). These storage proteins can be distinguished into two groups: the alcohol-soluble prolamins and the alcohol-insoluble glutelins. The determination of the presence of gluten in foodstuffs is mainly done by means of an immunochemical method called ELISA (enzyme-linked immunosorbent assay). This method is an approved Codex-standard [2] that stipulates the use of an R5 antibody assay [3]. The R5 ELISA determines the prolamin content of a sample, which is then converted into the gluten content by multiplication with a factor of 2. There are two versions, the sandwich ELISA for intact gluten proteins and the competitive ELISA for gluten peptides. A number of other assays are also available to quantify gluten in food. They not only include different antibodies but also diverse protein references for quantifying the gluten content.

The aim of this study was to investigate the chemical and immunochemical characterization of different protein references and to compare them with some of the ELISA kits and lateral flow assays used for determining gluten in food- stuffs.

Material and methods

Different ELISA kits for the detection of gluten in food were compared. A sandwich ELISA involving a monoclonal R5 antibody and the PWG gliadin as reference material (kit no. 1, Table 1) was used as the control, and the signal intensity was set to 100% [3]. In addition, three other sandwich ELISA kits (kits no. 2 - 4, Table 1) were used. Kit no. 2 used a monoclonal antibody according to Skerritt & Hill [4]. The antibodies used in kits no. 3 and 4 were monoclonal and polyclonal, respectively; however, no further information was available. In addition, two lateral flow assays (dip sticks), based on the R5 antibody, were tested.

Samples were extracted and analyzed according to the manufacturers' instructions. For gluten analysis, only protein references from wheat are. Either gliadin, the prolamin fraction of wheat, or 'gluten' consisting of gliadin and glutenin (the glutelin fraction of wheat) were used. Both products are not only used as references for the detection of gluten, but also for CD-specific medical analyses, e.g., the elucidation of the pathomechanism [5].

TABLE 1. *ELISA kits (no. 1 - 4) and ELISA sticks (no. 5 - 6) used in the study*

No.	Name	Producer	Type [a]	Antibody[b]	Extraction-method
1	RIDASCREEN® Gliadin (No. R7001)	R-Biopharm	S	R5/m	cocktail-solution
2	BIOKITS® Gluten assay kit (No. 802002Y)	Tepnel Research Products & Services	S	m	40% ethanol
3	Gliadin (No. ESGLI-48)	ELISA SYSTEMS	S	m	40% ethanol
4	Wheat Protein (Gliadin) (No. 181GD)	Morinaga Inst. of Biol. Science Inc.	S	p	β-mercapto-ethanol/buffer
5	RIDA® Quick Gliadin (No. R 7003)	R-Biopharm	-	R5/m	cocktail-solution
6	Operon Stick Gluten	OPERON	-	R5/m	60% ethanol

[a] *S = Sandwich*
[b] *m = monoclonal, p = polyclonal*

The following protein references were analyzed: ABCR 'Gliadin' (AB13 6288), ABCR 'Gluten from wheat' (AB13 6330), SIGMA 'Gliadin from wheat' (G 3375), SIGMA 'Gluten from wheat' (G 5004) and PWG gliadin [6]. Grains from the wheat cultivar 'Cubus', the rye cultivar 'Guttino', the barley cultivar 'Marthe' and the oats cultivar 'Typhon' were ground to wholemeal flours. The alcohol-soluble prolamins and the glutelins (alcohol-soluble after reduction of the disulfide bonds) were extracted from them and used as protein references [7]. 'Gluten' was washed out from the dough of the wheat cultivar 'Tommi' by means of a Glutomatic and afterwards freeze-dried [8].

The protein content (N x 5.7) of all references was determined with the Dumas method on a nitrogen analyzer. The content of protein fractions obtained by a modified Osborne procedure was analyzed by means of RP-HPLC [7].

Results and discussion

Reference proteins

As determined by means of the Dumas method, the crude protein content of the protein fractions was between 61% and 95% (Table 2). This had to be taken into account when these fractions were used for analytical purposes because different amounts of material were necessary to ensure the same amount of protein in the experiments.

TABLE 2. *Crude protein content (%) of the protein references* [a]

Reference	Protein	Reference	Protein	Reference	Protein
ABCR gliadin	78.4	wheat prolamin	88.7	wheat glutelin	74.0
Sigma gliadin	91.9	rye prolamin	90.0	rye glutelin	61.2
PWG gliadin [b]	94.6	barley prolamin	83.9	barley glutelin	75.7
ABCR „gluten“	71.5	oats prolamin	80.5	oats glutelin	67.3
Sigma „gluten“	77.0	„gluten“ cv. Tommi	88.1		

[a] *mean value of 3 determinations, average coefficient of variation = ± 0.6%*
[b] *from literature [9]*

For further characterization the commercially-available proteins were analyzed for their content of salt-soluble albumins/globulins, alcohol-soluble prolamins and alcohol-insoluble glutelins by means of a modified Osborne fractionation with subsequent HPLC analysis [7]. The results showed that all materials, even the samples declared as pure gliadin, contained considerable amounts (25.4% – 63.8%) of alcohol-insoluble glutelin [Fig. 1]. Thus, different materials (i.e., ABCR ‘Gliadin’ and SIGMA ‘Gluten’) had almost the same composition.

In summary, the protein analytical investigations showed that the protein references available on the market featured significant differences in terms of the content of crude protein as well as in the composition of Osborne-type protein fractions. Therefore, it can be concluded that the calibration of ELISA kits with these references would lead to different results in the determination of gluten.

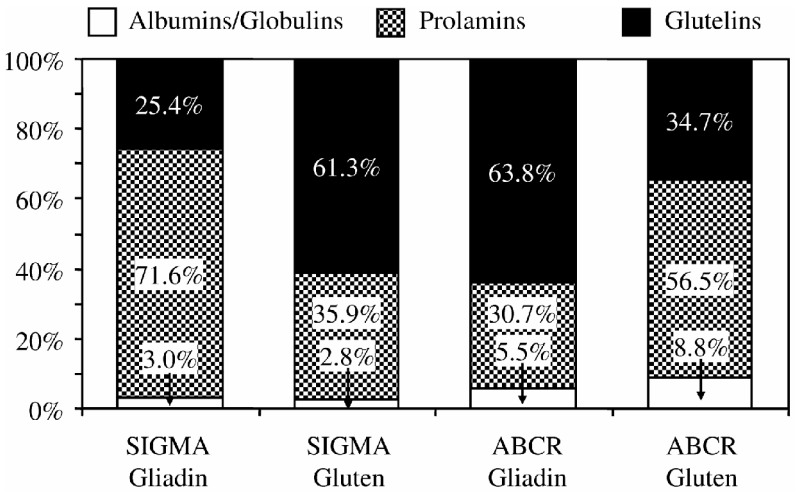

FIG. 1. *Content of protein fractions in protein references*

Comparison of ELISA kits

Before comparing the protein references, all ELISA kits were calibrated with PWG gliadin. Then, equal amounts of protein from the references (cf. Table 2) were analyzed by means of the different ELISA kits. Sample extraction was carried out according to the manufacturers' instructions by using the extraction agents given in Table 1. In the following tables, all data (%) is related to the results obtained with kit no. 1, whose signal intensity was set to 100%.

At first, the wheat, rye, barley and oats flours were analyzed (Table 3). In general, none of the kits was able to detect oats. In addition, the analyses of wheat, rye and barley flours showed that none among the kits no. 2 - 4 reached the values of kit no. 1. In particular, barley flour gave low responses (4% - 23%) compared to kit no. 1. The results of the isolated prolamin fractions were comparable to those of the flours (Table 4). Excepting for kit no. 2, all other ELISA kits provided higher values for the prolamin as compared to the glutelin fractions. The analysis of the commercially-available references showed considerable differences in the signal intensities ranging from 29% - 272% (Table 5), thus reflecting the different compositions of the materials.

TABLE 3. *Analysis of cereal flours with gluten ELISA kits. Signal intensity (%) as compared to kit no. 1 (control, set to 100%)* [a]

Kit No.	Wheat flour	Rye flour	Barley flour
1	= 100.0	= 100.0	= 100.0
2	52.9	28.3	3.6
3	78.2	93.6	5.4
4	72.3	27.0	23.0

[a] *mean value of 2 determinations; average coefficient of variation = ± 4.3%*

TABLE 4. *Analysis of prolamin and glutelin fractions from different cereals with gluten ELISA kits as compared to kit no. 1 (control, set to 100%)* [a]

Kit No.	Wheat		Rye		Barley	
	PROL[b]	GLUT [b]	PROL	GLUT	PROL	GLUT
1	= 100.0	13.0	= 100.0	39.9	= 100.0	12.9
2	40.9	43.0	37.7	44.2	4.9	3.0
3	55.9	10.4	68.0	55.5	5.2	1.3
4	59.1	14.6	15.6	5.4	34.0	6.4

[a] *mean value of 2 determinations; average coefficient of variation = ± 8.7%*
[b] *PROL = prolamins; GLUT = glutelins*

TABLE 5. *Analysis of different protein references with gluten ELISA kits as compared to kit no. 1 (control, set to 100%)* [a]

Kit No.	Gliadins			‚Gluten'		
	PWG	ABCR	Sigma	Tommi	ABCR	Sigma
1	= 100.0	56.3	68.8	= 100.0	128.7	272.2
2	95.0	119.8	160.8	43.0	142.8	57.9
3	37.0	33.5	28.6	54.2	61.7	58.6
4	97.4	124.6	122.0	103.1	112.5	133.6

[a] *mean value of 2 determinations; average coefficient of variation = ± 11.3%*

Comparison of lateral flow assays

For the easy and fast detection of gluten (prolamin) two lateral flow assays, based on the R5 antibody (ELISA dip sticks), were compared. They were applied to different flours and to all references listed in Table 2, according to the manufacturers' instructions. The band on the right-hand side of the stick was the control and indicated that the stick worked properly; whereas the band on the left indicated the presence of gluten. As a whole, both types of sticks were comparable (results not shown) with oats and oatmeal showing no reaction. In contrast, all the gliadin and 'gluten' references, the flours from wheat, rye and barley, as well as their prolamin and glutelin fractions, were positive (Fig. 2). The intensity of the left band correlated well with the amounts obtained with ELISA kit no.1 (Table 4). The glutelin fractions led to considerably weaker bands as compared to the prolamin fractions (Fig. 2).

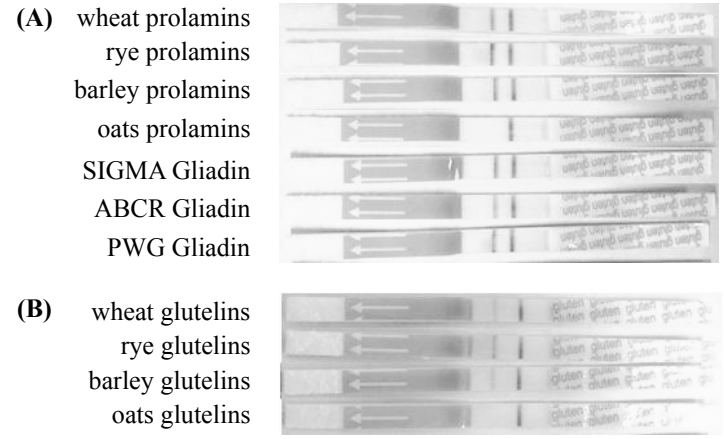

FIG. 2. *Analysis of prolamin and glutelin fractions with RIDA® QUICK Gliadin sticks (A) prolamins, (B) glutelins*

Conclusions

We concluded that the presence of gluten can be well detected by using lateral flow assays. However, it is not yet possible to obtain the quantitative information that is required for compliance with the threshold value of the Codex. Therefore, ELISA kits calibrated with reliable and well-characterized reference materials should be used. We recommend using PWG gliadin for this purpose. Commercially-available 'gluten' or 'gliadin' references show considerable differences in their composition and should, therefore, be checked against PWG gliadin before being used.

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Reactivity of different monoclonal antibodies towards gliadins and glutenins

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Abstract

The reactivity of three prominent antibodies was investigated after two-dimensional electrophoresis (2-DE) of a gliadin material (PWG gliadin) and transfer of the proteins via Western blot onto polyvinylidene fluoride (PVDF) membranes. Fluorescence labelling was used for the detection of the reacting and non-reacting proteins. For this purpose PWG gliadin was fluorescence labelled with Cy3 before 2-DE. After Western blot the PVDF membranes were incubated with anti-gliadin mouse antibodies 401.21, PN3 and R5 respectively. The reacting proteins were detected with a Cy5 fluorescence labelled anti-mouse antibody. Differential scanning at two specific wavelengths for Cy3 and Cy5 respectively showed the 2-DE pattern of the reacting and non-reacting proteins in the same membrane. Antibodies 401.21, PN3 and R5 each detected different protein sets of the gliadin material and thus can yield in different measurements of gluten amounts, when used in an ELISA assay for the determination of gluten. The findings help to explain why ELISA tests have been detecting different gluten amounts in the past, when different test kits were used.

Introduction

Coeliac Disease is one of the most frequent food intolerances worldwide, with a prevalence of 1 in 100 to 300 individuals [1]. The only way affected people can avoid symptoms is by adhering strictly to a life-long, gluten-free diet. Thus a sensitive and reliable detection method for gluten is needed. ELISA methods are taken to be the state-of-the-art analyses for detecting gluten in gluten-free food because of their sensitivity and specificity; however, they yield different results when different test systems are

used [2]. We investigated the reaction of three monoclonal antibodies often used for the detection of gluten, with proteins of a gliadin material separated by 2-DE.

Materials and methods

The gliadin-material had been extracted by means of 60% (v/v) ethanol from 28 of the most frequently-bred European wheat varieties [3]. It was named 'PWG gliadin' (this being the abbreviated form of 'Prolamin Working Group gliadin'), because it was initiated and produced by the Working Group on Prolamin Analysis and Toxicity.

The fluorescent labelling dye used was CyDye™ DIGE Fluor Cy™3 (Cy3), a minimal dye (GE-Healthcare, 25-8010-83).

The following primary antibodies were used:

1. Monoclonal antibody (mAb) 401.21: IgG1 mouse mAb, developed against gliadin by Skerritt & Hill [4], kindly provided by the company Vital Diagnostics Pty Ltd, Australia.
2. PN3-mAb: IgG1 mouse mAb, developed against a 19-mer peptide of A-gliadin by Ellis *et al.* [5], kindly provided by the research group of Prof. Dr. Paul Ciclitira.
3. R5-mAb: IgG2b mouse mAb, developed against secalin [6], kindly provided by Operon S.A., Cuarte de Huerva, Spain, via the late Dr. Enrique Méndez.

The secondary antibody used was ECL Plex goat anti-mouse IgG, labelled with fluorescent dye CyDye™ DIGE Fluor Cy™5 (Cy5) (GE-Healthcare, PA 45009).

Details about the labelling of the PWG gliadin, electrophoresis, Western Blot, antibody reaction and fluorescence scanning have been described in the published report of van Eckert *et al.* [7]. A very stringent washing regime and a high concentration of BSA in the blocking buffer were applied to avoid unspecific reactions. The allocation of proteins to gliadin and glutenin sub-groups was made by applying the apparent molecular weight known from our previous results and from other published data.

The efficiency of the blot and the consistency of the protein pattern were monitored at each stage of the procedure by means of fluorescence scanning of the Cy3-labelled proteins.

Results

The 2-DE protein pattern was the same throughout the entire process. The spots seemed slightly enlarged after the blot, probably due to diffusion. Some proteins in the migration area of ω gliadins and LMW glutenins were less intense after the completed antibody reaction.

The reacting proteins on the 2-DE maps clearly showed that each antibody detected a different set of proteins:

MAb 401.21 reacted mainly with proteins having an approximate molecular weight of 60,000 and above. It showed a reaction with HMW glutenin sub-units, presumably LMW glutenins, ω gliadins and - to a small degree - with α and γ gliadins. The most prominent reaction was observed in the HMW area.

PN3-mAb reacted mainly with proteins of an apparent molecular weight of 30,000 and higher, which corresponds to the size α gliadins.

R5-mAb reacted strongly with α and γ gliadins, especially those with a lower pI, with the reaction with γ gliadins being the strongest. It also reacted with proteins of a higher apparent molecular weight of 50,000 and around 75,000 and higher (probably ω gliadins).

Discussion

The fluorescence technique we used was more sensitive than the Coomassie Blue stain and allowed the scanning of protein patterns on gels and membranes at any time without change being introduced. CyDye DIGE minimal dyes are expected to add a single dye molecule to each protein and have a minimal effect on the charge and pI of the labelled protein [8]. The differential scanning of the Cy3-labelled gliadin and the Cy5-labelled antibody on the reacting proteins made it possible to measure both components in one membrane and thus avoid gel-to-gel variation, which is a great advantage to Coomassie Blue stained gels.

Some proteins in the ω gliadin and LMWglutenin area appeared to diffuse out of the membrane during the antibody incubation and washing regime. This is in agreement with the results of Hurkman & Tanaka [9], who observed a reduction in colloidal Coomassie Blue G-250 stained proteins when they were kept in water for 3 - 24 hours. Van den Broeck et al. [10] also noticed a reduction in ω gliadins, LMW glutenins and some α gliadins when Coomassie-stained gels were destained in 10% ethanol/7.5% acetic acid.

Our results showed that each antibody detected sets of different sub-types of gluten proteins to a different degree. This indicates that the amount of gluten detected is dependent on the antibody and on the reference material used. This had been assumed in the past but could not be illustrated until now.

MAb 401.21 reacts mainly with HMW glutenins. This explains why the gliadin preparations extracted by Wieser showed a relatively low reaction in gluten assays based on that mAb [2]. They were obviously very pure in terms of their gliadin content and did not contain many HMW glutenins. With our findings we can also explain why RM 8418, a gluten preparation from a Canadian spring wheat, exhibited a stronger response than the PWG gliadin in assays based on this antibody [3]. RM 8418 is composed of gliadins and glutenins, whereas the PWG gliadin had been extracted by means of 60% ethanol from wheat flour so that the gliadins are strongly enriched in this material. According to our results mAb 401.21 might be a candidate for the detection of HMW glutenins.

PN3-mAb seems to recognise distinctly α gliadins. This relates well with the fact that this mAb was raised against a peptide from A-gliadin, an α gliadin. It was suggested that this mAb reacted mainly with QQQPFP [5], which is found in α, but not in γ gliadins.

R5-mAb predominantly recognises the epitope with the QQPFP sequence [11]. It also reacts with homologous repeats such as QQQFP, LQPFP and QLPFP [12]. The QQPFP epitope occurs repeatedly in α, γ and ω gliadins. It has only one amino acid less than the main reactant QQQPFP of mAb PN3, and it occurs more often in γ and ω gliadins [13]. This is substantiated by our results in that mAb R5 showed a high reaction with γ gliadins. The diffusion of ω gliadins from the membrane during the incubation and washing steps of the antibody reaction might have diminished their response.

It is not possible to completely separate gliadins and glutenins from each other through extraction with aqueous ethanol [14]. Therefore PWG gliadin is enriched in regard to gliadins but also contains some glutenins. It has been found that glutenins pose the risk of coeliac toxicity as well [15]. The PWG gliadin is a valuable and representative material in determining gliadin content. If it is characterised clearly in regard of its glutenin content, it also can be used to determine glutenin content.

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A new enzyme-linked immunosorbent assay to detect the toxic gluten fragments and proteins involved in coeliac disease

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Coeliac disease (CD) is caused by inflammatory T-cell responses triggered by gluten fragments bound to the disease-associated HLA-DQ2 or HLA-DQ8 molecules. Gluten is a large protein family that can be subdivided into gliadins and glutenins and both these protein families have been shown to contain multiple immunostimulatory epitopes involved in CD. Many of the most immunogenic peptides, however, are found in the gliadins, in particular the α-gliadins [1]. Because of its unique properties, gluten is often used in the food industry, and gluten-free foods for CD patients must be produced under special conditions to guarantee their safety for consumption by patients. Levels of contamination may not exceed 20 mg/kg for foods prepared from naturally gluten-free ingredients, and 100 mg/kg for foods rendered gluten-free. Commercially-available enzyme-linked immunosorbent assays (ELISAs) are used to determine the level of gluten and the most frequently used are based on the R5 monoclonal antibody (mAb), which detects gliadin sequences not involved in CD. These kits are calibrated with a mixture of intact gluten proteins (both gliadins and glutenins) extracted with 60% ethanol from 20 different wheat varieties, called the Prolamin Working Group standard [2], which is, unfortunately, not a true standard as it cannot be reproducibly generated.

To overcome these shortcomings, the Leiden University Medical Center (Leiden, The Netherlands), in cooperation with EuroProxima (Arnhem, The Netherlands), has developed a novel competitive ELISA, termed Gluten-Tec®. This test is based on a mAb specific for a well-characterized T-cell stimulatory epitope of α-gliadin (α-20) in wheat [3]. This peptide does not represent a repetitive sequence but is present only once in α-gliadin proteins. The antibody specific for this peptide is thus well-suited for quantification. Moreover, the mAb also detects homologue sequences present in barley (hordein), rye (secalin) and their crossbred varieties and is thus also suitable for

detection of the presence of other harmful cereals [4]. A synthetic peptide is used for calibration, which allows an accurate and reproducible standardization. Moreover, as the test is a competitive ELISA, not only intact but also hydrolyzed proteins can be detected.

We have now tested the performance of the Gluten-Tec® ELISA kit by means of a collaborative study, in accordance to the guidelines of the *Association of Analytical Communities* [5]. Fifteen laboratories participated in this study, all of which were familiar with gluten testing and/or performing of ELISAs.

The study included four different food matrices, covering a wide range of hydrolysed and/or heat-treated food products, like two rice-based baby foods, one non-spiked and the other spiked with 5% wheat-based baby food; maize bread, spiked with 44.2 mg/kg of gliadin; two chocolate cake mixes, one non-spiked and the other spiked with 0.25% gluten-containing chocolate cake mix, and one beer.

The results have confirmed that Gluten-Tec® is suitable for the measurement of T-cell stimulatory epitopes over a wide range of concentrations and is suitable for gluten detection in the range required to guarantee the safety of food for consumption by CD patients. A manuscript describing the results of this study has been submitted to a peer-reviewed journal. The tests will be presented to the *Codex Alimentarius* as a preferred method for gluten analysis.

Acknowledgement

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Detection of toxic fragments from gluten using a new monoclonal antibody-based test

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Introduction

Coeliac disease (CD) is an immune-mediated enteropathy caused by the ingestion of gluten, a protein fraction found in certain cereals. CD occurs in genetically predisposed persons and leads to the destruction of the microscopic finger-like projections of the small intestine, called villi. The disease is triggered by the ingestion of peptides from wheat, barley, rye, and, in some cases, oats. CD currently affects roughly 1% of the world´s population, primarily adults. Immunotoxic gluten peptides, such as the fragment called 33-mer, which are resistant to the degradation of digestive enzymes, appear to trigger the coeliac syndrome. Homologues of this specific peptide were found in every food grain that is toxic to CD patients, but were absent in all non-toxic food grains [1]. A monoclonal antibody specific for a sequence occurring three times in the immunotoxic 33-mer has been developed [2, 3]. This work summarises the results of a new monoclonal antibody used in a lateral flow strip test that specifically recognises the pathogenic fragment of the gliadin protein present in gluten.

Methods

The semi-quantitative immunochromatographic strip test (AgraStrip® Gluten G12, Romer Labs UK) is based on a sandwich format. The reagents for the test and control line are immobilised on a nitrocellulose membrane. Toxic gluten fragments in the sample extract react with the anti-gliadin 33-mer monoclonal antibody, named G12, which is coupled to coloured microspheres. These are pre-dried on the strip showing a visible line when binding to immobilised anti-gliadin 33-mer monoclonal antibodies on the test line [2, 3]. The mix of conjugate moves through the membrane to the control line where anti-species specific antibodies, used for verifying the correct test performance, are sprayed. Several gluten-free samples and gluten-containing samples were analysed using the strip test. The results were confirmed by applying an ELISA, developed ourselves, using the monoclonal G12 antibody. We also applied a commercially-available gliadin ELISA test kit.

Results

The G12 antibody, specially developed to determine the toxic fractions present in gluten, was used for the semi-quantitative immunochromatographic strip test. The outstanding advantage of this new antibody in the strip test is that it enables the detection of the actual toxic fragment of gluten due to its very high sensitivity. The immunochromatographic test strip was compared with the ELISA methods by analysing a range of different food samples for their gluten content and results were found to be similar (Table 1).

TABLE 1. *Comparison of analysis of different food samples using AgraStrip® Gluten G12 (at10 ppm cut-off level) with ELISA results using G12 and R5 antibodies*

Type of food	Results (ppm gluten)		
	Results AgraStrip® Gluten G12	**Results G12 ELISA**	**ELISA R5**
Corn starch powder	> 10	18.3	21.5
Milk shake powder	> 10	191	220
Barbeque spice mix	> 10	23.3	19.2
Paprika powder	< 10	< 3	< 3
Wheat starch powder	> 10	166	203
Strawberry flavour	> 10	12	14.6
Pudding	> 10	36	28.6
Ham flavour	< 10	< 3	< 3
Glucose syrup	> 10	256	244
Rice milk	> 10	80	68.2
Sausage	> 10	113	98.2
Chorizo Sausage	> 10	> 100	155
Cured pork	< 10	< 3	< 3
Hamburgers	> 10	96	89
Gluten free cake	< 10	< 3	< 3
Aperitive snacks	< 10	< 3	< 3
Baby food	> 10	96	105
Gluten free biscuit	< 10	< 3	< 3
Gluten free bread	< 10	< 3	< 3
Chocolate	< 10	< 3	3.2
Ice cream	< 10	< 3	< 3
Cream	< 10	< 3	< 3

Limits of detection and cut-off

The detection limit of the AgraStrip® Gluten G12, after an incubation time of 10 min, is 15 ng/mL gliadin. This corresponds to 3 ppm of gluten in a sample using a 1:10 extraction and 1:10 dilution of the extract. Additional cut-off levels are applicable by using different dilutions after sample extraction (Table 2).

TABLE 2. *Detection limits for the AgraStrip® Gluten G12 using different sample extract dilutions*

	Liquid samples without extraction				**Solid samples with extraction**			
Dilutions	1:100	1:300	1:600	1:3000	1:10	1:30	1:60	1:300
Gluten detection limit ppm after 10 min incubation time								
Positive	> 3 ppm	> 10 ppm	> 20 ppm	> 100 ppm	> 3 ppm	> 10 ppm	> 20 ppm	> 100 ppm
Negative	< 3 ppm	< 10 ppm	< 20 ppm	< 100 ppm	< 3 ppm	< 10 ppm	< 20 ppm	< 100 ppm

Spiked food samples

Food samples spiked with PWG gliadin were analysed using AgraStrip® Gluten G12 with different sample dilutions, resulting in different cut off levels (Table 3).

TABLE 3. *Results using the AgraStrip® Gluten G12 with food samples spiked with PWG gliadin*

Matrix	**Level of fortification [ppm gluten] using PWG gliadin**	**Dilutions after extraction**	**Results AgraStrip® [ppm gluten]**
Baby food with cereals	blank	1:10	< 3 ppm
	20 ppm	1:30	> 10 ppm
		1:60	< 20 ppm
Syrup	blank	1:10	< 3 ppm
	20 ppm	1:10	> 3 ppm
	80 ppm	1:60	> 20 ppm
		1:300	< 100 ppm

Matrix	Level of fortification [ppm gluten] using PWG gliadin	Dilutions after extraction	Results AgraStrip® [ppm gluten]
Corn starch	blank	1:10	< 3 ppm
	40 ppm	1:60	> 20 ppm
	100 ppm	1:60	> 20 ppm
		1:300	> 100 ppm
Biscuits	blank	1:10	< 3 ppm
	40 ppm	1:60	> 20 ppm
	100 ppm	1:60	> 20 ppm
		1:300	> 100 ppm

FAPAS samples

FAPAS samples with assigned values were analysed using AgraStrip® Gluten G12 with different sample dilutions, resulting in different cut-off levels (Table 4).

TABLE 4. *Results using the AgraStrip® Gluten G12 with FAPAS proficiency samples*

Matrix	Assigned value of gluten [ppm]	Dilutions after extraction	Results AgraStrip® [ppm gluten]
Bread (FAPAS sample 2712A)	35.5 ppm	1:60	> 20 ppm
Bread (FAPAS sample 2712B)	26.9 ppm	1:60	> 20 ppm
Chocolate cookies (FAPAS sample 2722B)	15.7	1:30	> 10 ppm
Chocolate cookies (FAPAS sample 2726A)	41.2	1:60	> 20 ppm
Chocolate cookies (FAPAS sample 2733A)	7.12	1:10	> 3 ppm
		1:30	< 10 ppm
Chocolate Cake (FAPAS sample 2747A)	171	1:60	> 20 ppm
		1:300	> 100 ppm

Conclusions

According to the current Codex Alimentarius recommendations and the Commission Regulation (EC) No. 41/2009, food products can be labelled gluten-free if they contain less than 20 mg/kg gluten. The lateral flow test kit (AgraStrip® Gluten G12, Romer Labs UK), applying the monoclonal antibody named G12, can detect the toxic fractions of gluten from wheat and other cereals such as barley and rye.

The advantage of detecting the actual toxic fragment of prolamins is that it helps producers of gluten-free food and beverages to label the gluten content of their products correctly. This makes the product safer for consumers suffering from CD as they have no other option but to follow a life-long diet in which the intake of gluten is avoided.

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Analytical tools to detect gluten immunotoxic fractions in food based on monoclonal antibodies raised against the gliadin 33-mer peptide

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Introduction

Immunotoxic gluten peptides that are recalcitrant to degradation of digestive enzymes appear to trigger coeliac disease (CD). A 33-mer peptide from α-2 gliadin has been identified as a principal contributor to gluten immunotoxicity [1]. A gluten-free diet is the unique current therapy for CD patients; therefore, the characterization and quantification of the toxic portion of gluten in foodstuffs is crucial to avoid coeliac damage. Our aim was to develop immunological assays as novel food analysis tools to measure cereal fractions that are immunotoxic to CD patients.

Two monoclonal antibodies (mAb), G12 and A1, were developed against a highly immunotoxic gliadin 33-mer peptide [2]. In comparison to other ELISAs, those based on these antibodies showed a wider specificity for prolamins that are toxic to CD patients, along with a higher degree of sensitivity, accuracy, and reproducibility, than did the other ELISAs. Analyses of the available prolamin sequences revealed the potential epitopes in the immunotoxic prolamins of rye, wheat and barley [3]. Although G12 affinity for the 33-mer was superior to A1, the sensitivity for gluten detection was higher for A1. This observation correlated to the higher number of A1 epitopes found in prolamins than G12 epitopes. Both antibodies have been evaluated as analytical tools to develop different analytical techniques, including ELISA (competitive and sandwich) and immunochromatographic sticks. To satisfy the increasing demand from CD patients or their relatives and other potential non-specialized food-related professionals, we also developed a user-friendly immunochromatographic "sticks" version, called GlutenTox Home, with G12 mAbs showing consistent results compared to laboratory techniques for a broad range of food products.

Material and methods

All methods were used according to the manufacturer's instruction manual (Biomedal S.L. - GlutenTox ELISA Competitive [ref. KT-4758], GlutenTox ELISA Sandwich [ref. KT-5196], GlutenTox Sticks [ref. KT-4711]; Ingenasa S.L. - Ingezim Gluten Assay I-30.GLU.K2; R-Biopharm - RidaQuick Gliadin R7003). For the user-friendly gluten detection method (GlutenTox Home), the protocol is a simplified version of the GlutenTox Sticks instructions.

Food sample were ground with a clean food grinder, knife or hammer. With a graduated plastic spoon (1 mL), two level spoons of ground food was added to a bottle containing 10 mL of extraction solution (60% EtOH). For liquid samples, only one spoon (1 mL) was sufficient. For gluten extraction, the tube containing the sample was shaken vigorously for a total of 1min, then settled for 5 min to allow the solid rest to sink to the bottom of the tube. Using a platic pipette, a few drops were taken out from the upper extracted solution. Eight drops to detect 20 ppm were added to a tube containing 2 mL of dilution solution (1x PBT) The tube was mixed softly and 5 to 6 drops were added to a well at the tip of the immunochromatographic stick encased in a plastic cassette. After 10 min, the result was read. When a blue control line and a pink line appeared, the result was positive and above the chosen determined threshold (20 ppm, Codex Alimentarius norms). When a single blue line appeared, the result was negative and below 20 ppm and suitable for consumption by CD patients. The results were then compared with the results from an ELISA Sandwich G12.

Results and discussion

Comparison of R5 and G12 analytical techniques

Several hundreds food analyses were performed to compare G12-based analytical tools (ELISA Competitive as well as immunochromatographic sticks) to R5 antibody-related techniques. Our results showed concordance in the detection of gluten free food (< 20 ppm) in > 85% of the analyzed food from external analytical services as well as spiked samples (data not shown). However, certain discrepancies were found, and some of them are shown in Table 1. The main discrepancies were found in beer, probably because the ELISA Sandwich R5 could underestimate immunotoxic gluten peptides due to the abundance of single epitopes, which cannot be detected by a sandwich ELISA, although this is feasible by means of the ELISA competitive methods [4].

We detected two food samples containing soybean with no gluten-containing cereals in the ingredients list, that gave noticeable signals with R5 (Table 1). We also demonstrated, via different spiked samples, that the immunochromatographic sticks could consistently estimate gliadin content with different matrices when the dilution of extracted samples was adjusted (see examples in Table 1).

TABLE 1. *Gluten content analysis of food samples by means of methods based on G12 and R5 antibodies*

Spiked samples and analytical standard food	Sandwich ELISA R5 [ppm]	GlutenTox ELISA Competitive G12 [ppm]	GlutenTox Sticks G12 [ppm]
Chocolate cake mix (172 ppm)	231	254	> 100
Spiked maize bread (200 ppm)	171	140	> 100
Baby food (15 ppm)	12	11	> 10, < 20
Baby food (50 ppm)	39	42	> 20, < 100
Beer (160 ppm)	76	144	> 100
Beer (40 ppm)	12	31	> 20, < 100
Soybean sauce	11.5	< 3	< 3
Soybean flour	27.4	< 3	< 3

We also tested the capacity of different immunochromatographic sticks with either G12 or A1 antibodies to detect gliadin as well as the main immunotoxic peptide, the gliadin 33-mer. The immunochromatographic sticks with R5, A1 and G12 showed equivalent sensitivity in detecting gliadin (Table 2). However, R5 showed poor sensitivity in detecting 33-mer epitopes, since the detection limit was from 62 to 60,000-fold less sensitive than the A1 and G12 sticks, respectively. Equivalent differences were found by using ELISA methods (data not shown). These observations may be of particular relevance for hydrolyzed gliadin since the R5 may underestimate the presence of immunotoxic peptides.

TABLE 2. *Sensitivity of immunochromatographic dip sticks to PWG gliadin and to synthetic wheat gliadin 33-mer peptide*

Immunochromatographic dip sticks (lateral flow)	Detection limits (mg/Kg gliadin)	Detection limits (ng/mL gliadin 33-mer)
G12 mAb (GlutenTox Sticks G12)	1.5	0.01
A1 mAb (GlutenTox Sticks A1)	1	9.7
R5 mAb (R-Biopharm)	2.5	625

TABLE 3. *Analysis of gluten content of different commercial comestible products and food ingredients by ELISA Sandwich R5, ELISA Sandwich G12/G12 and GlutenTox Home*

Matrices	Results ELISA Sandwich R5	Results ELISA Sandwich G12/G12	Results GlutenTox Home
Corn starch	21.5 ppm	16.3 ppm	< 20 ppm
Sugar+milk	220 ppm	191 ppm	> 20 ppm
BBQ spices	19.2 ppm	23.3 ppm	> 20 ppm
Paprika	< 3 ppm	< 3 ppm	< 20 ppm
Wheat starch	203 ppm	166 ppm	> 20 ppm
Strawberry flavour	14.6 ppm	12 ppm	< 20 ppm
Pudding	28.6 ppm	36 ppm	> 20 ppm
Ham flavour	< 3 ppm	< 3 ppm	< 20 ppm
Glucose syrops	244 ppm	256 ppm	> 20 ppm
Rice milk	68.2 ppm	80 ppm	> 20 ppm
Sausage 1	98.2 ppm	113 ppm	> 20 ppm
Sausage 2	155 ppm	> 100 ppm	> 20 ppm
Cured loin of pork	< 3 ppm	< 3 ppm	< 20 ppm
Hamburgers	89 ppm	96 ppm	> 20 ppm
Cake (gluten free)	< 3 ppm	< 3 ppm	< 20 ppm
Aperitive snacks	< 3 ppm	< 3 ppm	< 20 ppm
Baby food	105 ppm	96 ppm	> 20 ppm
Biscuit (gluten free)	< 3 ppm	< 3 ppm	< 20 ppm
Bread (gluten-free)	< 3 ppm	< 3 ppm	< 20 ppm
Chocolate	3.2 ppm	< 3 ppm	< 20 ppm
Ice cream	< 3 ppm	< 3 ppm	< 20 ppm
Cream	< 3 ppm	< 3ppm	< 20 ppm

The robustness and the sensitivity of the immunochromatographic sticks encouraged us to develop a user-friendly kit for gluten detection in food (GlutenTox Home) without laboratory equipment. Various food samples with different types of matrices were selected for this study to assess whether a shorter and user-friendly method was satisfactory for estimating gluten content. Most of the results of this study revealed that despite the simplicity of the method, the consistency was high, with no discrepancies in a variety of food matrices (Table 3).

Conclusions

Our study suggests that mAb G12 and A1-based immunotechniques are robust and sensitive methods to evaluate the potential immunotoxicity of gluten in all types of food matrices that were tested. The R5-based products showed that they were at least two orders of magnitude less sensitive to the gliadin 33-mer peptide than G12 or A1-based methods. The user-friendly lateral flow test (GlutenTox Home), using the anti-gliadin 33-mer antibody, demonstrated that it could be useful for reliable gluten content estimations in a variety of food samples despite the simplicity and rapidity of the protocol.

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II. Clinical research reports

In-vivo quinoa feeding study

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Introduction

Dietary gluten can cause inflammation and histological deterioration of the small intestine in genetically predisposed individuals. An effective treatment for coeliac patients is to follow a strict gluten-free diet (GFD), however, gluten-free cereals are not widely available, are less palatable than other foods and can contain fewer nutrients than their gluten-containing counterparts. New gluten-free products that improve any of those qualities are a welcome alternative in the GFD. However, it is possible that traces of immunostimulatory peptides within the prolamin fraction of such alternative products can exacerbate coeliac disease (CD). Guidance with regard to potential toxicity can be sought in taxonomic classification and *in-vitro* experiments but, ultimately, feeding studies should confirm their suitability.

Quinoa is an Andean crop with excellent nutritional value and balanced amino acid content, and contains high levels of protein, fibre, vitamins and minerals in comparison to gluten-containing cereals. However, quinoa cultivars are known to contain up to 7% prolamin. Those cultivars with the highest content of putative immunostimulatory prolamins have been identified using *in-vitro* methods [1] . All cultivars had gluten levels within the recommended levels (below 20 mg/kg).

Confirmation about their suitability for coeliac patients is needed from an *in-vivo* study. Thus the aim of the present study was to examine the clinical, histological and immunological responses of adult coeliac patients before and after consuming quinoa as part of their usual GFD.

Materials and methods

Nineteen coeliac patients participated in the study: 2 males and 17 females with a median age of 59 years and a BMI of 23 kg/m^2, who were on a GFD for nine years and were all HLA-DQ2 positive. The study was approved by the Ethical Committee at St

Thomas' Hospital, London, and all patients gave written informed consent before participating in the study. All participants were diagnosed adult coeliac patients on a GFD for at least one year. Participants were excluded if they had any medical condition considered sufficiently serious to interfere with the study or to constitute an unacceptable risk to them.

Participants were asked to consume 50 g of pre-weighted quinoa every day for six weeks as part of their usual gluten-free diet. They were free to choose the cooking method but it was recommended that they consumed quinoa flakes for breakfast as porridge or pancakes. Patients were given a diary card [2] to record any symptoms of diarrhoea, abdominal pain, increase in bowel sounds or vomiting during the entire study period. Serological coeliac screening tests, including IgA anti-gliadin (AGA), IgA anti-TTG (AtTG) and IgG and IgA anti-endomysium (EMA), were used to monitor compliance with the GFD. A full blood count, and liver and renal profiles were used to monitor the health status of patients. Iron, tests of vitamin B12, serum folate and a lipid profile were used to determine the effects of quinoa on the GFD. All tests were analysed before and after the study.

Ten treated coeliac patients provided duodenal biopsies for morphometric measurements at the beginning and end of the study. The normal range for these parameters are between 5:1 and 3:1 for VH:CD, between 29 to 34 nm for SECH and between 10% and 30% for the IEL count. CD can be diagnosed when VH:CD is less than 3:1, SECH is below 29 nm and IEL above 30%. Alterations in these parameters can be used as a reliable indicator of exacerbation of the condition. The VH:CD ratio was measured in 10 different areas with at least 10 measurements of this ratio per biopsy on H&E stained slides; the SECH in at least 30 randomly-selected enterocytes in the mid-third of villi per biopsy on H&E stained slides and the IEL in 10 different areas per biopsy stained for CD3+ cells.

Results

Gastrointestinal symptoms were differentiated according to four categories (diarrhoea, abdominal pain, increased bowel sounds and vomiting) and graded daily (0 = none, 1 = mild, 2 = moderate and 3 = severe). Ten patients did not report any symptoms. Nine patients reported symptoms ranging from mild to moderate during the first two weeks of the study. Most of them were mild abdominal pain, followed by a mild increase in bowel sounds and diarrhoea. This might be due to an increase in dietary fibre, as reported in other feeding studies [3]. Serological coeliac screening test results were within normal levels.

Duodenal biopsies from 10 patients were assessed randomly and blindly, before and after consumption of quinoa, by applying three morphometric parameters (VH:CD, SECH and IEL). Results indicate that the mean values of VH:CD rose from slightly

below-normal levels (2.8:1) to normal levels (3:1), similar results were observed for SECH, with values rising from 28.76 to 29.77 μm. IEL values decreased from slightly abnormal (30.3) to just below normal (29.7). Although a positive trend was observed (increased VH:CD and SECH, decreased IEL), no significant differences were seen in any of the measurements.

The mean values of VH:CD (3:1) and SECH (29.77 μm) at the end of the study were at the lower end of the normal range (3:1 to 5:1 and 29 to 34 μm, respectively) which was to be expected in a group of coeliac patients with a wide range of time on a GFD (1 to 33 years) as it could take more than two years to achieve normal or quasi-normal morphometric parameters after initiation of treatment (GFD) [4]. In some patients, full recovery is never achieved for various reasons, including hidden sources of gluten in their diets, complication of the disease (development of refractory CD) or other unexplained causes [5].

The other morphometric parameter, IEL count, was, conversely, on the higher end of normality (10% to 30%) after quinoa consumption (29.7%). All median values for blood tests at the beginning and at end of the study were within the appropriate normal range, expecting for total cholesterol and LDL, the values for which were slightly higher than the recommended 4 and 2 mmol/L, respectively [6]. Untreated coeliac patients tend to have lower cholesterol levels [7] which, after treatment with a GFD, tend to increase. One of the mechanisms that could contribute to this increment is the increased absorption of saturated dietary fat after a GFD is started [8].

The total cholesterol in the study population reduced from 4.6 to 4.3 mmol/L, and LDL fell from 2.46 to 2.45mmol/L, while the reduction in HDL from 1.8 to 1.68 mmol/L was significant ($p = 0.05$) after eating quinoa. This reduction in cholesterol confirms the results of an early study in which induced hypercholesterolemia in mice improved strongly by feeding them with quinoa [9]. Although, the cholesterol values were still slightly higher than the recommended level and there was a reduction in HDL, it is clear that patients could benefit from eating quinoa. However, more studies are needed to determine whether this positive trend continues over a longer period of time.

Conclusions

The addition of quinoa to the GFD of 19 adult coeliac patients did not cause exacerbation of the disease. Gastrointestinal symptoms were either absent during the study or mild in some patients in the first two weeks of the study. Most patients continued eating quinoa after the study. This could be interpreted as an early indication that quinoa is well tolerated among coeliac patients. However, a larger number of participants and a validated method to assess psychological well-being as well as a wider range of gastrointestinal symptoms would be required to confirm this interpretation.

In addition, the positive trend towards improvements in some serological parameters, particularly the hypocholesterolemic effects, require further evaluation.

Overall, the data suggest the likely suitability of quinoa as part of a GFD, which had hitherto been assumed, without supporting clinical data.

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Immunogenicity of two oats varieties

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Introduction

Coeliac disease (CD) is characterised by a derangement of both the adaptive and the innate immune response to gliadin. Some gliadin peptides that are deamidated by tissue transglutaminase (e.g., A-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules and induce an adaptive Th1 proinflammatory response [1]. Other gliadin peptides (e.g., P31-43, P31-49) are not recognized by T cells and induce an innate immune response mainly mediated by IL15 [2].

To date there is still little information on the immunogenic properties of cereals others than wheat. Protein fractions of barley and rye, which are known to be toxic to coeliac patients, are able to activate gliadin-reactive T-cell lines obtained from the intestine of coeliac patients, showing that these cereals, like wheat, can induce an adaptive immune response [3]. Nothing is known regarding the ability of these cereals to induce an innate immune response.

Recently, several studies have focused on oat as a cereal that can be introduced into the CD diet. *In-vivo* studies in children and adults seem to indicate that oats can be tolerated by CD patients [4]. However, Lundin *et al*. [5] reported that some patients on an oat-containing diet had abdominal discomfort and one patient developed villous atrophy and dermatitis. Moreover it has been shown that gliadin-reactive T-cell lines obtained from the intestine of coeliac patients may respond to protein fractions of oats [3].

Although oats have been the object of several studies in the recent times, some questions about their toxicity still remain unanswered. One issue is the individual reactivity of CD patients, as some CD patients are responsive to oats. Another issue is the possibly different toxicity of oats varieties.

The aim of our study was to investigate immunological properties of two oats varieties, *Avena genziana* and *Avena potenza*, in terms of their safety for coeliac patients.

Methods and results

IL15 expression

High levels of IL-15 are present in intestinal mucosa of coeliac patients in the active phase of the disease. By means of immunohistochemistry, we investigated the IL15 expression both in the epithelium and *lamina propria* of the intestinal mucosa of CD patients on a gluten-free diet (GFD) before and after 24 hours of *in-vitro* treatment with the medium alone or with PTG, PT-genziana or PT-potenza. Before culture, IL15 was highly expressed in the villi and crypts epithelium of small intestinal mucosa of CD patients on a GFD. The cytokine was mainly found in the apical region of the enterocytes. This pattern sometimes presented as a patchy distribution. IL15-positive cells were also detected in the *lamina propria.*

After *in-vitro* culture for 24 hours with the medium alone, IL15 staining decreased both in the villi and crypts epithelium. *In-vitro* culture in the presence of PTG, induced an increase in IL15 expression in villi and crypts epithelium and in the *lamina propria* as against tissue cultured with the medium alone. On the contrary, after 24 hours of culture with PT-genziana and PT-potenza, we did not observe any significant increase in IL15 staining in villi and crypts epithelium or in the *lamina propria*.

Intraepithelial lymphocytes infiltration

A significant increase of CD3+ intraepithelial lymphocytes was seen in biopsies of CD patients on a GFD, which were cultured for 24 hours in the presence of PTG (32 ± 18 cell/mm epithelium) and also with PT-potenza (23 ± 12 cell/mm epithelium), as against those cultured in medium alone (13 ± 6 cell/mm epithelium). By contrast, no differences were noted in the number of intraepithelial CD3+ cells in biopsy specimens treated with PT-genziana (16 ± 8 cell/mm epithelium).

Mononuclear cell activation in lamina propria

CD25 expression in the *lamina propria* was evaluated to find evidence of activated mononuclear cells. Consistent with previous results, the expression of the above-mentioned marker was significantly higher in biopsy specimens from CD patients on a GFD after 24 hours *in-vitro* treatment with PTG (49 ± 32 mm2 of *lamina propria*) in comparison to the tissue cultured using the medium alone (13 ± 8 CD25+/mm2 of *lamina propria*). Such an increase was not observed in fragments cultured with PT-genziana (23 ± 17 CD25+/mm2 of *lamina propria*) or PT-potenza (21 ± 17 CD25+/mm2 of *lamina propria*).

T-cell lines and IFNγ production

We analyzed the long-term ability of prolamins from the oat varieties potenza and genziana to stimulate intestinal T-cells lines established from eight DQ2 positive coeliac individuals and raised against deamidated PT-gliadin. All iTCLs displayed marked IFNγ production when stimulated with deamidated PT-gliadin: median: 5.7 ng/ml (range 0.9-12.5). Although iTCLs from 3 patients cross-reacted with PT-genziana, and only when it was deamidated, on the whole the IFNγ induced by the oat genziana was much lower than the level observed in responses to gliadin: 1.9 ng/ml (range 0-10) ($p < 0.05$). With the exception of one patient, in which a positive response to PT-potenza was elicited, with an average of IFNγ production of 1.4 ± 10 ng/ml, PT-potenza did not induce significant response in any of the patients analyzed.

Conclusions

In this study, several *in-vitro* tests and biological assays were used to investigate the immunological effects of two oat varieties, *Avena genziana* and *Avena potenza*, in CD patients. These tests were designed to investigate the adaptive and the innate immune response of the coeliac intestine to oats prolamins in comparison to gliadin peptides. Oats prolamine peptides were not able to induce, in CD enterocytes, an increase in IL15 or in D25 positive cells. Overall, the results of the *in-vitro* tests suggest that the two oats varieties we studied are virtually safe for CD patients. These results are consistent with those of clinical studies demonstrating that oats are generally well tolerated by the majority of CD patients [4, 6]. We propose this experimental approach, based on an extensive *in-vitro* evaluation of biological and immunological proprieties, to screen cereal varieties selected by breeding or modified for coeliac patients, before conducting *in-vivo* studies.

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Gluten toxicity, how to get rid of it?

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In 1993 Lundin and colleagues first described the presence of gluten-specific T cells in small intestinal biopsies of coeliac disease (CD) patients [1]. A large number of studies have since established that such T cells can be specific for a large and diverse array of peptides derived from the gliadins as well as the glutenins ([2-5], references in [6]).

Invariably, these peptides can trigger T-cell responses only when bound to the disease predisposing HLA-DQ2 or HLA-DQ8 molecules. This provides an explanation for the well established association between these HLA-molecules and disease development. It also became evident that many of these peptides require modification by the enzyme tissue transglutaminase, a modification that introduces negative charges into gluten peptides, thus enhancing the binding of these peptides to either HLA-DQ2 or HLA-DQ8 [8]. Not only wheat is off-limits to CD patients: barley and rye are also known to contain a variety of proteins that are just as harmful as the gluten proteins from wheat [8, 9]. Oat seems an exception as it is tolerated by most patients, partly due to a low content of gluten-like prolamin proteins [8-10].

Thus, CD patients usually have T cells specific for an array of gluten and gluten-like peptides that originate from all types of gluten proteins and homologues in other cereals. At present the introduction of a gluten-free diet is the only but highly-effective treatment option. This diet, however, has several drawbacks. It is relatively cumbersome, difficult to adhere to, expensive and is deficient in several nutrients and fibers. Many patients feel insecure, especially when eating out or while travelling. Thus, there is an unmet need for alternatives to the gluten-free diet.

With the identification of the harmful sequences in gluten and gluten-like proteins it has become possible to initiate studies aimed at reducing or eliminating the toxicity of such proteins and/or wheat. Early studies indicated that substantial differences existed between wheat varieties regarding their "toxicity profile" [11-13]. Some of those could be attributed to differences in the genetic make-up of the three genomes that comprise the complex hexaploid bread wheat, the A-, B- and D-genome [12, 13]. More recently we embarked on a large-scale study to map the toxicity of the α-gliadins, based on the observation that the α-gliadins are among the most immunogenic gluten proteins and

contain four well-characterized peptides involved in CD [2, 4, 5]. We analyzed over 3,000 α-gliadin genes in the database to determine the full extent of the natural variability that is present in these genes and in the known T-cell stimulatory peptides in these proteins in particular [14]. The results indicated that many natural variants of these immunogenic α-gliadin peptides exist; an example is given in Fig. 1.

Sequence	Occurrence in database
PFPQPQLPY	986
PFLQPQLPY	106
PFSQPQLPY	190
PFPHPQLPY	29
ETC	

FIG. 1. *Many natural vairants of known immunogenic alphy-gliadin peptides*

We could classify all identified variants as belonging to one of the three genomes based on differences in the complete gene sequences. Moreover we synthesized all variants of the four known T-cell peptides that we had identified, and tested these for binding to HLA-DQ2 or HLA-DQ8 as well as for recognition by T cells derived from small intestinal biopsies of CD patients ([14], Fig. 2). The results demonstrated that no α-gliadin proteins exist that lack all T-cell stimulatory epitopes ([14], Fig. 2). Based on these results it can be concluded that it would be impossible to generate CD-safe wheat through conventional breeding programs. Substantial differences, however, were observed between the genes encoded by the three genomes: while the D-genome α-gliadins are by far the most toxic, the B-genome encoded α-gliadins are the least toxic and the A-genome encoded genes have an intermediate toxicity profile.

	DQ2-α1	**DQ2-α2**	**DQ2-α3**	**DQ8-α1**
A	+	-	+	-
B	-	-	-	+
D	+	+	+	+

FIG. 2. *No alpha-gliadin proteins exist that lack all T cell stimulatory epitopes*

Close examination of the results, however, indicated that there are natural variants of all T-cell stimulatory epitopes that do not induce T-cell responses. For example, the A-genome encodes a variant of the DQ2-α2 epitope that is not immunogenic: while the sequence of this epitope on the D-genome is PQPQLPY**P**Q, the A-genome encodes PQPQLPY**S**Q and this P to S substitution results in a peptide that does not induce T-cell responses [14]. Similarly, natural variants of the other three T-cell stimulatory peptides have been identified that lack T-cell stimulatory properties.

These results allow a novel approach to eliminate gluten toxicity: while the D-genome α-gliadin gene encodes four toxic epitopes, it is possible, , by combining the genetic information of the A- and B-genome encoded α-gliadins, to generate a new gene that encodes a that is not toxic for CD patients ([14], Fig. 3). At the peptide level, we have provided proof of principle for this approach and we envisage that similar approaches can be taken to generate non-toxic variants for the other gliadin and glutenin proteins in wheat gluten. In this respect the high molecular weight glutenins are of particular interest as these, to a large extent, determine the baking properties of gluten. Genes encoding such safe-gluten proteins could be introduced into safe cereals for production of safe-gluten that could be used for the fabrication of gluten-free foods with markedly enhanced quality in terms of nutritional value, taste and baking properties.

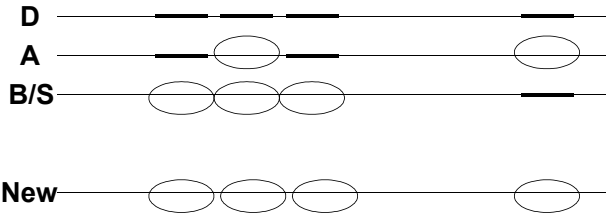

FIG. 3. *New non-toxic gluten gene*

Acknowledgement

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Antibodies in the diagnosis of coeliac disease in young children

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Introduction

Assays meassuring IgA antibodies against tissue transglutaminase (anti-tTG) and endomysium (EmA) and IgG antibodies against deamidated gliadin peptides in serum have a high sensitivity and specificity for coeliac disease (CD) in children [1, 2]. However, in young children (up to two years of age), antibodies against native gliadin (anti-nGli) are still assumed to have a higher diagnostic accuracy.

In young children, IgA-EmA are reportedly less sensitive, and have maximum values of 89% [3-8]. The sensitivity of IgA-anti-tTG ranged between 83% and 90% [1?, 2?, 6, 8, 9]. The specificity of IgG-anti-nGli was only 77% at high sensitivity [5]. It has been claimed that IgA-anti-nGli was the best means for detecting CD in young children [6], as it has a sensitivity between 82% and 97% and a specificity between 88% and 94% [1?, 2?, 5-8]. Little data is available to date on the test performance of antibodies against deamidated gliadin peptides are rare until now, particularly in very young children. We investigated the validity of antibodies against deamidated gliadin peptides and compared the results with those from other antibody tests.

Patients and methods

The sera of 173 children below three years of age were retrospectively examined. The patients were recruited from the Municipal Hospital "St. Georg" in Leipzig (Germany), the Departement of Laboratory Medicine of the University Hospital in Leuven (Belgium), and also from the University Children's Hospital of Leipzig, Munich, and Giessen (Germany). The patients comprised 39 children with CD and 134 controls (93

females and 80 males, mean age 1.57 years, 95% range 0.6 to 2.9 years). Sera samplings wa done at the time of duodenal biopsy. All patients following a normal (gluten-containing) dietand were biopsied to suspicion of CD or other gastrointestinal disorders. The intestinal pathology of all CD patients was in accordance with Marsh 2 or Marsh 3 criteria.

IgA and IgG antibodies against deamidated gliadin (analogous fusion) peptides (anti-GAF3X), anti-nGli, anti-tTG, and IgA-EmA were measured (without prior knowledge of the diagnosis) by means of the test kits from EUROIMMUN Medizinische Labordiagnostika Lübeck, Germany. The analyses were performed by EUROIMMUN.

Data were evaluated by means of the receiver operating characteristic (ROC) analysis. The area under the ROC-curves (AUC) was calculated. Differences between ROC-curves were evaluated by pairwise comparison according to a chi-square analysis. An error probability of less than 0.05 was considered statistically significant. Non-inferiority testing was performed if there was no statistically-significant difference. For non-inferiority testing, the lower end of the 90% confidence intervals (CI) of differences between AUCs was considered. Non-inferiority was assumed if the lower end of the 90% CI of the difference was not below 0.01 (zone of diagnostic in-difference of 1%).

For the cut-offs suggested by the manufacturer, diagnostic accuracies, sensitivities, and specificities were calculated. The significance of differences ($p < 0.05$) was evaluated by applying McNemar's test in which applying 2 x 2 contingency tables containing the number of patients are classified as correct or incorrect. For non-inferiority testing, we compared two tests showing the percentage of children with false-negative, false-positive, or false positive and false-negative results, for the oeliac patients, the control children, and for all patients, respectively. The z-test was applied for calculation of the 90% CI of differences in proportions. Non-inferiority was assumed if the lower end of the 90% CI of the difference in proportions was not below 0.01.

Results

The results are summarized in Table 1. After examining all the antibody tests, we found that the AUC of IgG-anti-GAF was the highest (0.960). The AUC of IgG-anti-GAF was significantly higher than that of IgA-anti-nGli and non-inferior to that of IgG-anti-nGli, of IgG-anti-tTG and of IgA-EmA. In the case of IgA-anti-GAF, it was not possible to draw any conclusions interms of significance of differences or non-inferiority.

TABLE 1. *Performance of different antibody tests in children aged up to three years*

	IgA-anti-GAF	IgG-anti-GAF	IgA-anti-nGli	IgG-anti-nGli	IgA-anti-tTG	IgG-anti-tTG	IgA-EMA
Test No.	1	2	3	4	5	6	7
Cut-off (U/L)	25.0	25.0	25.0	25.0	20.0	1.0	10
AUC	0.934	0.960 ^{3,4,6}	0.908	0.935	0.951	0.929	0.945
Sensitivity	0.769 ^{4,6}	0.872 ^{3,6}	0.692	0.949	0.872	0.462	0.897
Specificity	0.993 ^{3,4,5,7}	0.963 ^{4}	0.955	0.731	0.948	0.985	0.955
Accuracy	0.942 ^{3,4,6}	0.942 ^{3,4,6}	0.896	0.780	0.931	0.867	0.942
PPV at PrTP of							
0.01	0.510	0.191	0.118	0.034	0.144	0.238	0.168
0.02	0.678	0.323	0.213	0.067	0.254	0.387	0.290
0.05	0.844	0.552	0.411	0.157	0.468	0.619	0.513
0.10	0.920	0.722	0.596	0.282	0.650	0.775	0.690
0.20	0.963	0.854	0.768	0.469	0.807	0.885	0.834
0.50	0.990	0.959	0.930	0.779	0.943	0.969	0.952
NPV at PrTP of							
0.01	0.998	0.999	0.997	0.999	0.999	0.995	0.999
0.02	0.995	0.997	0.993	0.999	0.997	0.989	0.998
0.05	0.988	0.993	0.983	0.996	0.993	0.972	0.994
0.10	0.975	0.985	0.965	0.992	0.985	0.943	0.988
0.20	0.945	0.968	0.925	0.983	0.967	0.880	0.974
0.50	0.811	0.882	0.755	0.934	0.881	0.647	0.903

Superscripts denote significant differences to tests with the respective numbers, subscripts denote non-inferiority to the tests. Significant differences and non-inferiority only indicated for comparison of IgA- and IgG-anti-GAF with other antibody tests. Non-inferiority tests only performed if there was no statistically significant difference. PPV positive predictive value, NPV negative predictive value calculated for different pre-test probabilities (PrTP).

In the analysis in which the manufacturer's cut-off in diagnostic accuracy was found for IgG-anti-GAF, IgA-anti-GAF and of IgA-EmA was applied, the highest (0.942 in all three antibody tests). The accuracy of IgG-anti-GAF was significantly higher than that of IgG-anti-nGli and of IgG-anti-tTG, but non-inferior to IgA-anti-nGli. The accuracy of IgA-anti-GAF was significantly higher than that of IgG-anti-nGli, IgA-anti-nGli and IgG-anti-tTG. A comparison between IgA- and IgG-anti-GAF yielded inconclusive results.

The specificity of both anti-GAF-tests was at least non-inferior to the IgG-anti-nGli assay. Non-inferiority to the IgA-anti-nGli assay could only be shown for the IgA-anti-GAF test, although the estimate of the specificity of both anti-GAF tests was higher than that of the two anti-nGli assays.

Whereas the sensitivity of the IgG-anti-GAF test was significantly higher that of the IgA-anti-nGli assay, a comparison with the IgG-anti-nGli assay proved inconclusive. The sensitivity of the IgA-anti-GAF test was even significantly lower than that of the IgG-anti-nGli assay and a comparison with the IgA-anti-nGli assay proved inconclusive.

In a pre-test probability of up to 10%, negative predictive values were higher than 0.98 for IgG-anti-GAF, IgG-anti-nGli, IgA-anti-tTG, and IgA-EmA. With increasing pre-test probabilities, negative predictive values for all assays, except that for the IgG-anti-nGli test decreased below 0.93. Positive predictive values were lowest for IgG-anti-nGli, regardless of the pre-test probability.

In one patient, none of the tested antibodies was positive (Marsh 3c). In three other patients (all Marsh 3a), only IgG-anti-nGli were elevated. In one of these three patients (HLA-DQ8 positive) secretory IgA deficiency could be demonstrated.

Summary and conclusion

Our study did not only comprise children up to two years of age (as it was described in previous reports cited above) but also included children in the age group between two and three years. We included the latter group in order to obtain a sample size sufficient for statistical evaluation. The inclusion of older children, however, may hide the special features of antibody diagnostics in the young age group.

Only 23% of the patients studied were coeliacs. Thus, the accuracy is strongly determined by specificity. The accuracy of both anti-GAF assays was at least non-inferior to that of the two conventional anti-nGli-tests. Both, accuracy and specificity of the two anti-GAF tests are saytisfactory as they are at least non-inferior to the two assays for anti-nGli (excepting in the comparison of the IgG-anti-GAF assay with the IgA-anti-nGli-test, whichwas inconclusive).

We conclude that the measurement of IgG-anti-GAF, IgG-anti-nGli, IgA-anti-tTG, and IgA-EmA results in the highest negative predictive values in children below three years of age. In a pre-test probability up to 10%. the negative predictive values of these tests are at least 98.5%. Anyway these assays, however, the positive predictive value of the IgG-anti-nGli test is very low, which renders this test unsuitable as a diagnostic tool.

A selection bias in our results cannot be excluded. First of all, biopsies are more likely to be performed in symptomatic patients with positive results for CD specific antibodies such as IgA-EmA and IgA-anti-tTG, which favours these tests. On the other hand, most children in this study had not been re-challenged with gluten in order to confirm the diagnosis of CD as per the ESPGHAN criteria [10]. Therefore, we cannot guarantee that the three patients with enteropathy and positive IgG-anti-nGli, but with negative results for all other antibody tests, were true CD patients. Since the specificity of anti-nGli antibodies is low, mucosal lesions due to infections or allergy might have been taken as indicators of CD. Confirmation or exclusion the diagnosis of CD is therefore crucial and should be done by means of follow-up, exclusion of IgA deficiency, HLA-analysis and/or gluten provocation.

Due to the low number of coeliac patients, the power of our study was low. More patients with clearly defined diagnostic criteria, especially children below two years of age are required in future studies.

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Signalling pathways controlling the TG2 expression in the small intestinal mucosa

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Introduction

Transglutaminase 2 (TG2) is a multifunctional protein located in several cell compartments such as the cytoplasm, the nuclei and the mitochondria. TG2 works as a G protein in transmembrane signalling and has protein disulphide isomerase and protein kinase activity. It is, however, the catalytic activity leading to either deamidation or formation of ε-(γ-glutamil) lysine crosslinks between proteins (termed transamidation) that has received major attention [1]. This biological activity can either occur on the cell surface or in the extra cellular matrix, where it crosslinks matrix components that promote tissue stability, cell adhesion and cell migration. Altogether, these functional properties have important roles in tissue repair, inflammation and apoptosis. The dysregulation of TG2 is involved in the pathogenesis of various human disorders, including neurodegenerative and autoimmune diseases [2, 3].

Recent research has therefore focussed not only on the role of TG2 in the pathogenic mechanisms of disease but also on the therapeutic use of its pharmacological inhibition [4]. Particularly in coeliac disease (CD), selective deamidation of glutamine residues in gliadin/glutenin-derived peptides leads to higher affinity binding to the HLA predisposing alleles DQ2 (A1*0501, B1*0201) and DQ8 (A1*0301, B1*0301), thus leading to greater gliadin-specific T cell stimulation. In addition, TG2 catalyzes a covalent linkage between the glutamine-rich gliadins/glutenins and itself or with other acceptor proteins [5].

The small intestinal mucosa in untreated CD patients is characterized by the presence of several proinflammatory cytokines such as TNFα, γIFN, IL-6, and IL-15, among others. Although signal transduction pathways of proinflammatory cytokines have been investigated in different systems, their role in TG2 expression in the intestinal mucosa has not yet been assessed.

The aim of this study was to evaluate the induction of TG2 expression by proinflammatory cytokines and to assess the signalling pathways operating in the small intestine.

Methods

Cell line: Caco-2 cells (human colon adenocarcinoma) were used as a model of human intestinal epithelia.

Biopsy samples: Intestinal biopsies were taken as part of routine procedure to diagnose CD in adult patients suffering from different gastrointestinal symptoms. The diagnosis was reached by means of histological examination, serology and analysis of clinical presentation. The present study was approved by the HIGA San Martin hospital's ethical committee, La Plata, Argentina.

Cytokine treatments and inhibition of signalling pathways: Caco-2 cells and biopsy samples were either stimulated only with proinflammatory cytokines for 24 h (TNFα 10 ng/ml endogen RTNFA1, γIFN 200 UI/ml BD554617, IL-1 10 ng/ml BD 551838, IL-6 10 ng/ml and IL-15 20 ng/ml BD 554630) or in the presence of inhibitors of signalling pathways, such as JNK/C-Jun pathway (SP600125 20 μM calbiochem), MAPK-p38 pathway (SB203580 10 μM calbiochem), PI3K pathway (wortmanine 10 μM calbiochem), AKT pathway (Ly294002 2 μM sigma) and NF-κB pathway (sulfasalazine 10 μM sigma and Bay11-7082 1 μM sigma).

Quantitative real-time PCR: Using the sequence of TG2 gene reported by our group (Genbank AY675221), a specific pair of primers was designed to amplify a 243 bp TG2 fragment. Real-time PCR was performed to determine the TG2 RNAm level after different treatments. Quantitative PCR was performed in the Cycler real-time PCR (BioRad). β-actin was the housekeeping gene used for normalisation.

Results and Discussion

In active CD, proinflammatory cytokines, such as TNFα, γIFN, IL-1, IL-6 and IL-15, are actively produced in the small intestinal mucosa. *In-vitro* assays were used to evaluate the role of these cytokines on the modulation of TG2 expression. Caco-2 cells were incubated for 24 h with TNFα, γIFN, IL-1, IL-6 and IL-15 and the expression of TG2 was determined by quantitative real-time PCR. Figure 1A shows that γIFN and TNFα are the most potent inducers of TG2 expression; in particular, γIFN produced an 18-fold increase.

Taking into consideration that γIFN and TNFα are typical Th1 cytokines and abundant in the small intestine mucosa in active CD, we further evaluated the expression of TG2 on Caco-2 cells stimulated by both cytokines. The results of the quantitative PCR showed that the expression of TG2 in Caco-2 cells treated with TNFα+γIFN was higher (25,7) than the value estimated by adding the fold increase values for TNFα and γIFN (21,2) (Fig. 1B). Therefore, the incubation of TNFα+γIFN produced a synergistic effect on TG2 induction.

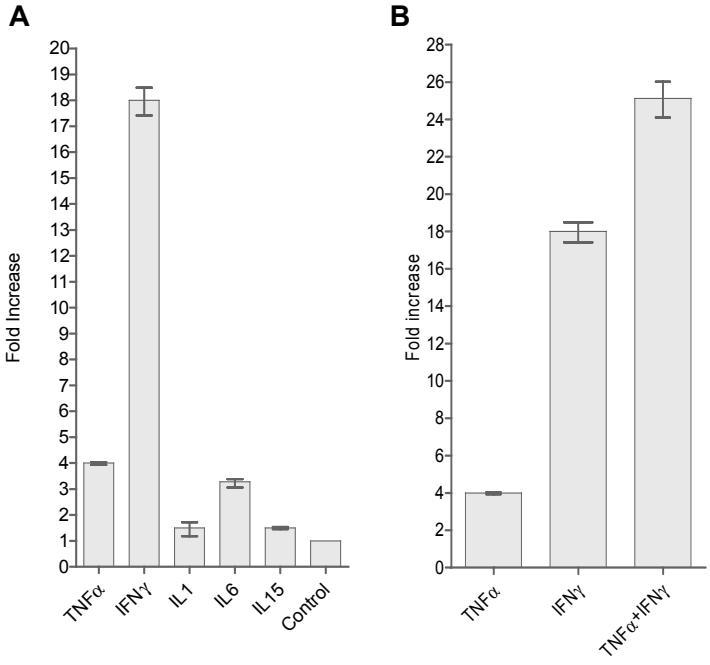

FIG. 1. *Induction of TG2 by proinflammatory cytokines.*
TG2 expression was determined by quantitative RT-PCR in Caco-2 cells incubated with proinflammatory cytokines.
A - TG2 expression in Caco-2 cells estimulated for 24 h with TNFα, γIFN, IL-1, IL-6 and IL-15.
B - Synergistic effect of the incubation of TNFα+γIFN on the induction of TG2. Results were expressed as fold increase of TG2 determined by comparison with the housekeeping gene (bactin) and referred to unstimulated cells (value = 1).

Quantitative RT-PCR showed that TG2 was strongly induced by TNFα+γIFN, both in untreated CD patients as well as controls (Fig. 2). The signalling pathways observed in Caco-2 cells are also active in the small intestine. The induction was much higher in active CD than in the control samples, suggesting that signalling pathways are activated to a higher extent in untreated CD patients, due to the chronic inflammatory process, or, instead, that CD patients tend to respond more strongly to this stimulus.

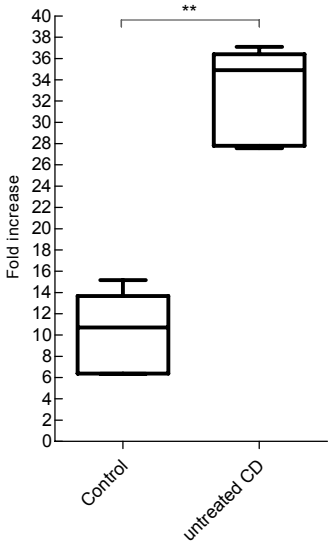

FIG. 2. *Induction of TG2 by TNFα+γIFN in the small intestinal mucosa Intestinal biopsies from untreated CD patients (n = 6) and control individuals (n = 7) were incubated for 24 h with TNFα+γIFN . Fold increases of TG2 were determined by comparison with the housekeeping gene (βactin) and referred to unstimulated tissue from the same patient (p < 0.001).*

The TG2 promoter contains binding sites for several transcriptions factors, with NFkB being one of the most important due to the multiplicity of its biological effects. A set of inhibitors were used to assess the signalling pathways involved in TG2 induction by TNFα and γIFN. Caco-2 cells were incubated with TNFα, γIFN or TNFα + γIFN, in the presence of the following inhibitors: SP600125 (C-Jun), SB20358 (MAPKp38), wortmanine (PI3K), Ly294002 (AKT), sulfosalazine (NFkB) or Bay 11-7082 (NFkB).

When Caco-2 cells were stimulated with TNFα, the expression of TG2 was completely blocked in the presence of SB203580, sulfasalazine and Bay-117082 and only partially by SP600125 (Fig. 3). In contrast, wortmanine, Ly294002, sulfasalazine and Bay-117082 blocked TG2 induction when Caco-2 cells were stimulated by γIFN. Therefore, NFkB pathway was central to the induction of TG2 as either TNFα or γIFN were completely blocked by sulfasalazine and Bay-117082. However, both cytokines induced different cascades since induction of TG2 by TNFα was selectively blocked by SB203580 (inhibitor of p38MAPK), while wortmanine (inhibitor of PI3K) and Ly294002 (inhibitor of AKT) selectively blocked the induction of TG2 by γIFN.

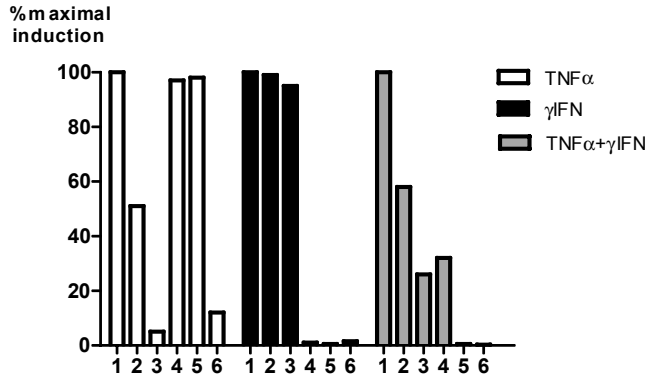

FIG. 3. *Signalling pathways involved in the induction of TG2 in Caco-2 cells stimulated with TNFα and γIFN.*
Percentage of maximal expression of TG2 in Caco-2 cells incubated with TNFα, γIFN or TNFα+γIFN in the presence of 1. no inhibitor, 2. SP600125, 3. SB20358, 4. wortmanine, 5. Ly294002, and 6. sulfosalazine. Results were expressed as the percentage of fold increases of TG2 as determined by comparison with the housekeeping gene (βactin) and referred to cells with no inhibitor (value = 100).

Inhibition with sulfosalazine (NFkB) or Ly294002 (AKT) produced a reduction in TG2 induction in CD tissue samples as well as in control individuals incubated with TNFα + γIFN (not shown), thus indicating that the same signalling pathways operate in the small intestine mucosa.

Conclusions

Different proinflammatory cytokines were used to modulate the expression of TG2 in an in-vitro model of human enterocytes (Caco-2 cells). Among the cytokines tested, γIFN was the most potent inducer of TG2 expression. A synergistic effect on the expression of TG2 was observed in cells stimulated with a combination of TNFα+ γIFN. Similar results were also observed when intestinal biopsies were treated with these cytokines. Since TNFα and γIFN are present in the small intestine in untreated CD patients, the higher induction of TG2 by these cytokines must be considered as part of the pathogenic mechanism in CD.

Specific inhibitors selectively blocked signalling pathways involved in the induction of TG2 by TNFα and γIFN. These effects were also observed in biopsy samples of the small intestine mucosa. Since signals producing upregulation of TG2 can participate in several disease processes and particularly in CD, the knowledge of the molecular pathways triggering the induction of TG2 constitutes valuable information for the development of new therapeutic approaches.

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III. Alternative and novel therapies

Gluten sensitivity: The new kid on the block of the gluten spectrum disorder

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Wheat, rice, and maize are the most widely consumed food grains in the world. Wheat, the most widely-grown crop, is immensely diverse, with over 25,000 different cultivars having been produced by plant breeders worldwide. Much of the world production of wheat is consumed by humans, after being processed into bread and other baked goods, pasta and noodles and, in the Middle East and North Africa, bulgar and couscous. In addition, the broad availability of wheat flour and the functional properties of gluten proteins provide the rationale for their wide use as an ingredient in food processing.

Gluten is the main structural protein component of wheat. Possibly, the introduction of gluten-containing grains, which occurred about 10,000 years ago with the advent of agriculture, represented a "mistake of evolution" that created the conditions for human diseases related to gluten exposure, the best known of which are mediated by the adaptive immune system: wheat allergy and coeliac disease (CD). In both conditions, the reaction to gluten is mediated by T-cell activation in the gastrointestinal mucosa. However, in wheat allergy it is the cross-linking of IgE by repeat sequences in gluten peptides [e.g. Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe] that triggers the release of chemical mediators, such as histamine, from basophils and mast cells [1]. In contrast, CD, which affects approximately 1% of the general population, is an autoimmune disorder, as indicated by specific serologic markers, most notably serum antitissue transglutaminase (tTG) autoantibodies, by the autoimmune enteropathy that characterizes this condition, and by autoimmune co-morbidities.

Besides CD and wheat allergy, there are cases of gluten reactions in which neither allergic nor autoimmune mechanisms are involved. This is generally defined as *gluten sensitivity* (GS) [2-5]. Some individuals, who experience distress when eating gluten-containing products and show improvement when following a gluten-free diet, may have GS rather than CD. GS patients are unable to tolerate gluten and develop an adverse reaction when eating gluten that usually, and differently from CD, does not lead to small intestinal damage. While the gastrointestinal symptoms in GS may resemble those associated with CD, the overall clinical picture is generally less severe

and is not accompanied by the concurrence of tTG autoantibodies or autoimmune disease. Typically, the diagnosis is made by exclusion, and an elimination diet and "open challenge" (i.e., the monitored reintroduction of gluten-containing foods) are most often used to evaluate whether health improves with the elimination or reduction of gluten from the diet.

A number of *in-vitro* studies have confirmed the cytotoxicity of gluten's main antigen, gliadin. Gliadin has agglutinating activity, reduces F-actin content, inhibits cell growth, induces apoptosis, alters redox equilibrium, and causes a rearrangement of the cytoskeleton through the zonulin pathway and the loss of tight-junction (TJ) competence in the gastrointestinal mucosa [6-9]. The diversity of gluten-induced conditions is in line with the notion that the immune system reacts to and deals with the triggering environmental factor, gliadin, in distinct ways.

The symptoms in GS may resemble those associated with CD [10,11], but with a prevalence of extra-intestinal symptoms such as ataxia, behavioural changes, bone or joint pain, muscle cramps, leg numbness, weight loss, chronic fatigue (Table 1). While the class II MHC haplotype HLA-DQ2 and DQ8 are present in almost all CD patients, these genes are present in only about 50% of patients with GS, a percentage slightly higher than in the general population. This suggests a reduced level of involvement of MHC-dependent, adaptive immune responses in GS relative to CD [10,11]. In the last decade, several studies have shown that there are signs and symptoms associated with non-coeliac gluten sensitivity, particularly in the neuropsychiatric disorders. Persons with schizophrenia have higher than expected titres of anti-gliadin antibodies, which are related to CD and GS, whereas gluten free diet seems to improve the behaviour of a subset of children with autism spectrum disorders (ASDs). However, currently there are no laboratory biomarkers specific for GS. Usually the diagnosis is done by exclusion, and an elimination diet of gluten-containing foods, followed by an "open challenge" (monitored reintroduction of gluten-containing foods). These are most often used to evaluate whether health improves with the elimination or reduction of gluten from the diet.

Since specific biomarkers need to be developed for specific diagnosis, we propose that the definition of GS is based on those cases of gluten reaction in which both allergic and autoimmune mechanisms have been ruled out (diagnosis by exclusion criteria).

More specifically, these are cases with:

- Negative immuno-allergy tests to wheat;
- Negative CD serology (EMA and/or tTG) and in which IgA deficiency has been ruled out;
- Negative duodenal histopathology;

- Possible presence of biomarkers of gluten immune-reaction (AGA+);
- Presence of clinical symptoms that can overlap with CD or wheat allergy symptoms;
- Resolution of the symptoms following implementation of a GFD.

Based on the aforementioned concept, it is clear that this new form of gluten reaction within the gluten spectrum disorder needs to be better defined through controlled studies to define its nature, pathogenesis, proper diagnosis, and management.

TABLE 1. *Gluten sensitiviy: What is the magnitude of the Problem?*

The CFCR Experience (2004-2010)	
Nr. of the patients seen at the CFCR clinic	5,896
Nr. of patients fulfilling criteria for GS	347
Prevalence in our cohort	1:17 (6%)
Symptoms	Abdominal pain: 68% Eczema and/or rash: 40% Headache: 35% "Foggy mind": 34% Fatigue: 33% Diarrhea: 33% Depression: 22% Anemia: 20% Numbness legs/arms/fingers: 20% Joint pain: 11%

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Presumptive safety for coeliac patients of wheat-baked goods rendered gluten-free during sourdough fermentation

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Coeliac disease (CD) is an inheritable disorder of the small intestine that affects approximately 1% of the world population, and is characterized by an inflammatory response to ingested wheat gluten and similar proteins in rye and barley, leading to small intestinal mucosa injury and nutrient malabsorption [1, 2]. The gluten network is formed by interactions between gliadins (prolamins) and glutenins (glutelins) when flour and water are mixed. Gluten is unique among the main dietary proteins in that it contains approximately 15% proline and 35% glutamine aminoacid residues. The high concentration of glutamine and, especially, praline, prevents proteolysis by gastric and pancreatic enzymes and results in the building up of oligopeptides, at the level of the small intestine, which are toxic to CD patients [1].

During the past decades, cereal food technology has changed to such an extent that it has modified the dietary habits of entire populations that had previously been naïve to massive gluten exposure. Moreover, baked cereal goods are currently manufactured by means of fast processes: thus, long-time fermentation through sourdough, a cocktail of acidifying and proteolytic lactic acid bacteria, has been almost completely replaced by the use of chemical leavening agends and/or baker's yeast. Cereal components (e.g., proteins) are not degraded during production under these technological conditions [1].

CD can be controlled only by maintaining a strictly gluten-free diet (GFD); however, good compliance is difficult to achieve and some nutritional deficiencies have emerged [3]. Other therapeutic options, such as the oral supplementation with microbial oligopeptidases, have been proposed [1].

Recently, several studies were carried out in the author's laboratory, with the aim of showing the capacity of proteolytic enzymes, mainly peptidases, from selected sourdough lactobacilli, to degrade gluten during food processing [3-10]. In particular, several studies showed that pools of lactic acid bacteria (sourdough lactobacilli and commercial probiotic preparation) under specific processing conditions (long-time fermentation) had the capacity to markedly hydrolyze and detoxify

wheat and rye prolamins fractions, as shown by two-dimensional electrophoresis and toxicity screening based on agglutination tests on K562(S) cells [4-8]. The semi-liquid pre-fermentation of wheat flour was another indispensable condition to fully exploit the potential of enzymes from sourdough lactobacilli [4, 5, 9]. The processed flour obtained by means of the biotechnological protocol set up in these pioneer studies was used for manufacturing bread in mixture with containing gluten-free flours, and for breads used for an *in-vivo* double-blind acute challenge of CD patients [5]. Thirteen of the 17 coeliac patients had values of intestinal permeability that did not differ significantly from the baselines values [5]. The same approach as those used for wheat flour and bread was adapted for rye and pasta making, and similar results were obtained [6-8]. In all the cases, although acute *in-vivo* and many *in-vitro* tests were carried out, only a marked decrease in the gliadin fraction was shown, and further studies were addressed for getting the complete detoxification of the wheat flour [4-8].

Subsequently, it was shown that a food grade biotechnology consisting of a combination of 10 selected sourdough lactobacilli with fungal proteases, routinely used in bakery, decreased the residual concentration of gluten in wheat flour, during food processing, to below 10 ppm [9]. Fungal proteases are indispensable for generating polypeptides of intermediate dimensions (e.g., 4-40 amino acids) from native proteins, which are suddenly transported inside the lactobacilli cells and hydrolyzed through the complex peptidase system of lactobacilli [10]. It was demonstrated that a large number of intracellular peptidases (e.g., PepN, PepO, PEP, PepX, PepT, PepV, PepQ and PepR) are involved in the hydrolysis of the immunogenic polypeptides to free amino acids [10].

After lactobacilli fermentation, the wheat flour was spray dried. Bread, pasta or sweet baked goods could be manufactured with the wheat flour rendered gluten-free. Its use has indubitable economic, nutritional, social and sensory advantages as compared to its naturally gluten-free counterpart currently used in the GFD. Indeed, as shown by means of sensory analysis, the properties of baked sourdough goods, manufactured with wheat flour rendered gluten-free were almost comparable to the full-gluten counterpart and were highly superior with respect to gluten-free baked goods ([9]. Based on the above results, two independent clinical challenges were carried out, which involved the daily administration of 200 g of sweet baked goods made with wheat flour rendered gluten-free (corresponding to ca. 10 g of native gluten) [2, 11]. Eight coeliac patients in remission were enrolled in the first challenge [11].

Even though an intestinal biopsy, showing the typical picture of flat mucosa, is still considered the gold standard for diagnosing CD, all the routine complementary analyses (haematology, serology and intestinal permeability analyses) were carried out in this study [11]. One patient discontinued the trial after 15 days and another after 30 days, mainly due to compliance difficulties during daily consumption. All the other

patients showed normal values for haematology, serology and intestinal permeability during the 60 days of the challenge [11]. In the second trial, 5 patients were challenged by having to consume baked goods made with processed flour [2]. None of the patients had clinical complaints over the 60 days; and they did not produce anti-tTG (anti-transglutaminase) antibodies, nor were modifications of the small intestinal mucosa visible, and the Marsh grade remained unchanged during the challenge (Fig. 1) [2].

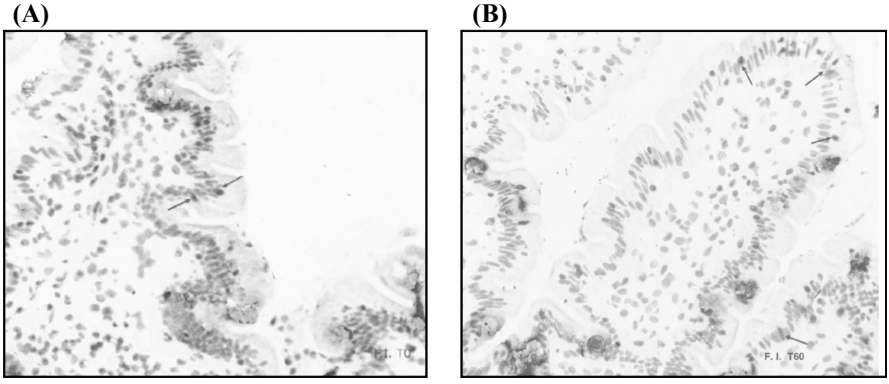

FIG. 1. *Density of γδ+ intraepithelial lymphocytes in a jejunal biopsy from a coeliac patient at the beginning (panel A) and after 60 days of challenge (panel B) with wheat-baked goods rendered gluten-free*

In conclusion we can state that the challenges done demonstrated that a wheat flour-derived product was not toxic after being administrated over 60 days to CD patients. Nevertheless, this length of time might not have been sufficiently long to evaluate toxicity in all coeliac subjects, who may occasionally show a different sensitivity to gluten [2, 11].

New and longer trials have been planned to demonstrate the safety of baked goods manufactured by applying this promising and recently rediscovered biotechnology.

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Transamidation of wheat: An enzyme strategy to detoxify gluten

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Introduction

Coeliac disease (CD) is characterized by the activation of intestinal gluten-specific $CD4^+$ T cells. In particular, gluten becomes a better T cell antigen following deamidation catalyzed by tissue transglutaminase (tTG) [1]. We reported that a preventive transamidation of gliadin by a single incubation of wheat flour with microbial TG (mTG) and lysine methyl ester (K-CH_3), completely inhibited the IFN-γ expression of intestinal gliadin-specific T cell lines from CD patients [2].

More recently, we showed that a protracted intake of this transamidated gluten was tolerated in a subset of CD patients [3].

The present work investigates the effects on the adaptive immune response *in vitro* and the technological properties of transamidated wheat flour and semolina.

Methods

A wheat flour suspension, depleted of the albumin/globulin fraction was subjected to two sequential steps of transamidation with mTG (ACTIVA®WM; 81-135 U/g; Ajinomoto Foods Hamburg, Germany) and lysine ethyl ester (K-C_2H_5; NutraBio.com, Middlesex, NJ, USA) at 30 °C. Soluble proteins and gliadin fractions were recovered and analyzed by SDS-PAGE.

Intestinal T-cell lines were isolated from three HLA-$DQ2^+$ adult CD patients and incubated with various preparations of pepsin/trypsin (pt)-digested and tTG-deamidated gliadin. Culture supernatants were analyzed for IFN-γ protein secretion by ELISA.

Bread wheat flour or durum wheat semolina was suspended in water containing 8 U/g mTG and 20 mM K-C_2H_5. Incubation was performed in two steps: first step, 2 h at 30 °C followed by centrifugation (1100 g 10 min); second step, 3 h at 30 °C with fresh enzyme and K-C_2H_5. The suspension was finally centrifuged to recover dough. A bread baking procedure was tested with 500 g of dough mixed with 3.0 g table salt and 10.0 g baker's

yeast. A pasta making machine was used to make pasta (spaghetti) with a length of 200 mm and a thickness of 1.75 mm. The pasta was dried according to the following schedule: 90 °C, 83% relative humidity (rh) 3 h; 63 °C, 73 % rh 1 h; 40 °C, 70% rh 90 min, room temperature 48 h. The prolamin content in transamidated products was determined by means of R5 ELISA (Istituto Ricerche Agrindustria, Modena, Italy).

Results

The transamidation reaction generated a soluble form of gliadin along with transamidated insoluble gliadin (Fig. 1A). Notably, we confirmed that transamidated insoluble gliadin was inactive, whereas soluble gliadin was still active when challenged *in vitro* with intestinal T-cell lines from CD patients (Fig. 1B).

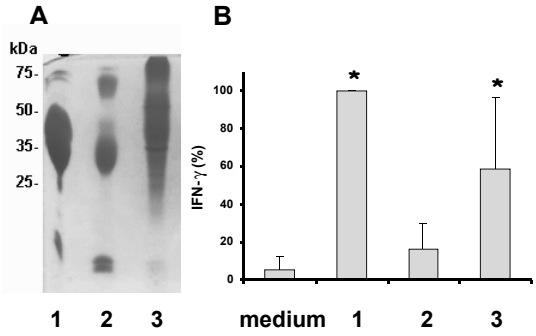

FIG. 1. *A) SDS-PAGE of gliadin fractions isolated from transamidated flour.*
B) IFN-γ produced in vitro by intestinal T-cell lines isolated from CD patients (n = 3) following challenge with gliadin.
1 - native gliadin; 2 - transamidated insoluble gliadin;
*3 - soluble gliadin; * - different from medium, P < 0.05 (ANOVA test).*

We found that the gluten content in transamidated bread, determined by means of R5 ELISA, drastically decreased from 1102.7 mg/kg, after the single step, to 5.8 mg/kg after the two-step reaction (Table 1). A similar result was obtained for transamidated semolina (Table 1), suggesting that prolamins were extensively masked following a two-step transamidation process. The transamidated wheat bread had a wheat-like flavour, a brown crust colour and a crumb structure similar to the control bread (Fig. 2A). Our findings, however, showed that the specific volume was lower than in the control bread (2.21 vs. 2.69 ml/g, transamidated vs. control). Similarly, a dried pasta was produced with transamidated semolina (Fig. 2B). The water uptake of transamidated pasta following cooking was comparable to that of untreated pasta (146% vs. 149%; transamidated vs. control).

Table 1. *Gluten concentrations of final products*

Final products	Gluten (mg/Kg)
Untreated wheat bread (ICC137)	72000 ± 1000
One-step transamidated bread (R5 ELISA)	1102.7 ± 34.0
Two-step transamidated bread (R5 ELISA)	5.8 ± 0.8
Untreated pasta (ICC137)	115000 ± 1150
Transamidated pasta (R5 ELISA)	13.7 ± 2.3

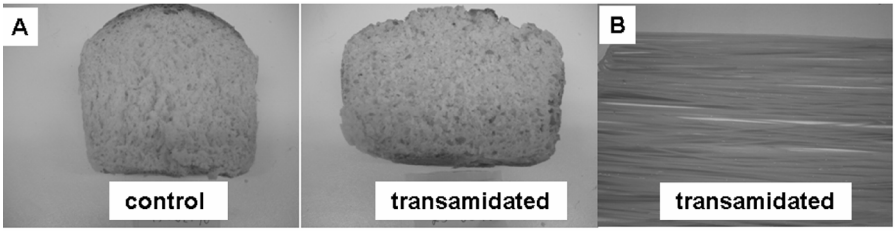

FIG. 2. *A) bread prepared by using untreated (left) or two-step transamidated wheat flour (right). B) dried pasta (spaghetti) prepared by using a two-step transamidated wheat semolina*

Conclusions

This study shows that transamidation generated a soluble form of gliadin that continued to be immunologically active, along with inactive insoluble gliadin. The two-step transamidation processing of wheat flour or semolina eliminated this fraction and completely blocked the immune recognition of wheat prolamins by R5 monoclonal antibody. This treatment did not hamper the main technological properties of gluten, as good quality bread and dried pasta were produced. The *in-vivo* safety of the two-step transamidated wheat flour for coeliac patients is under investigation.

Aknowledgements

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Peptidases for degradation of gluten and possible use in dietary therapy

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Introduction

Coeliac disease (CD) is a common inflammatory disease of the small intestine that is triggered by the storage proteins of wheat, rye, barley and, possibly, oats in genetically-predisposed individuals. This group of proteins (wheat gliadins and glutenins, rye secalins, barley hordeins, oat avenins) is collectively referred to as 'gluten'. The current treatment for CD is a strict, lifelong, gluten-free diet to prevent chronic enteropathy and reduce the risk of lymphoma and carcinoma. The maximum daily intake of gluten should not exceed 20 mg, which corresponds to around one hundredth of a slice of bread. This dietary restriction, however, is a big challenge for CD patients and may lead to poor compliance or inadvertent intake of gluten. There is, therefore, an urgent need to develop safe and effective therapeutic alternatives and, based on the advanced understanding of the pathomechanism of CD, non-dietary therapies have been devised during the last decade. Examples include modified flours that have been depleted of immuno-dominant gluten epitopes, or a decrease in intestinal permeability by blocking the epithelial zonulin receptor, inhibition of intestinal transglutaminase activity, inhibition of proinflammatory cytokines and inhibition of peptide presentation by HLA-DQ2 antagonists (recently reviewed by Schuppan *et al.* [1]). Intense clinical trials will be necessary to ensure the effectivity of such alternatives.

Peptidases for gluten degradation

The structural features unique to all CD-toxic proteins are amino acid sequence domains rich in glutamine (Q) and proline (P) [2]. The high proline content, particularly in the repetitive sequence motifs, renders these proteins resistant to complete proteolytic digestion by human gastrointestinal enzymes. Consequently, large proline- and glutamine-rich peptides are accumulated in the small intestine; a striking example is the '33-mer' peptide from α-gliadins (sequence: LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), which has been shown to be resistant to gastric and pancreatic peptidase-mediated digestion [3]. Such peptides penetrate into the subepithelial lymphatic tissue, and, depending on the amino acid sequences, induce two different immune responses: the rapid innate response and

the slower adaptive response [1, 2]. Hence, one strategy to prevent those peptides from reaching the lymphatic tissue has been to make use of special peptidases that cleave large toxic peptidases into small non-toxic fragments with less than nine amino acid residues ('oral enzyme therapy'). In addition, peptidases have been recommended for decreasing the level of gluten proteins and peptides in raw material for food production (e.g. starch) or food (e.g. beer) before ingestion by CD patients ('detoxification of gluten'). Bacteria, fungi and germinating cereals have been proposed as sources for gluten-degrading peptidases.

Koshla's and Sollid's groups were the first to make use of bacterial peptidases (prolyl endopeptidases, PEPs) for the detoxification of gluten peptides [3, 4]. They are expressed in various microorganisms such as *Flavobacterium meningosepticum*, *Sphingomonas capsulata*, and *Myxococcus xanthus* [5]. The effectiveness of these PEPs, however, were limited by restrictions on the length of their substrates, their sensitivity against low pH (< 5) and pepsin, and the long duration necessary to completely digest gluten peptides [1]. However, PEPs from *Sphingomonas capsulata,* in combination with endopeptidase B2, a glutamine-specific peptidase from barley, has been shown to have an effective synergistic potential for the detoxification of gluten proteins and peptides [6, 7]. The combined enzymes are currently being tested in phase 2a clinical studies to evaluate safety and efficacy.

Koning's group studied a prolyl endopeptidase from *Aspergillus niger* (AN-PEP), which is suitable for both the removal of gluten from food as well as from oral supply [8, 9]. Its advantages are, in comparison to bacterial PEPs, the resistance to low pH and to pepsin activity, degradation of both proteins and peptides, and, in addition, strains of the genus *Aspergillus* have a food-grade status. It was demonstrated that AN-PEP degraded gluten to non-toxic fragments by digesting a food matrix in an *in vitro* gastrointestinal system [9, 10]. Phase 1 clinical trials did not indicate any toxicity of the enzyme; and phase 2 trials are in preparation.

Sourdough *lactobacilli* have been shown to produce specific peptidases that hydrolyse proline-rich peptides [11]. Together with fungal peptidases they are capable to degrading gluten in sourdough so that treated sourdough can be used as a gluten-free ingredient in gluten-free formulations for baked goods.

Cereal peptidases have long been known to degrade storage (gluten) proteins during the germination of seed to provide the embryo with amino acids. Therefore, they were tested for their capability to detoxify gluten peptides by means of extensive fragmentation [12]. A highly active peptidase fraction extracted from germinated rye bran has been shown to cleave CD-toxic peptides into fragments with less than nine amino acids very quickly and at different pH values (Fig. 1). Moreover, they are able to degrade intact gluten proteins. Peptidase preparations from germinated cereals have distinct advantages as compared to PEPs from microorganisms. They contain mixtures of endo- and exopeptidases with optimal specificities for gluten degradation. The enzymes

derive from a naturally safe food source and no genetic engineering is necessary. Their production is part of a well-established technological process (malting, beer production) and is rather simple and cheap. Therefore, peptidase preparations from germinated cereals are promising candidates for the detoxification of gluten-containing foods, as was recently shown by the application on beverages such as kwas and malt beer [13], and, possibly, for the oral therapy of CD patients.

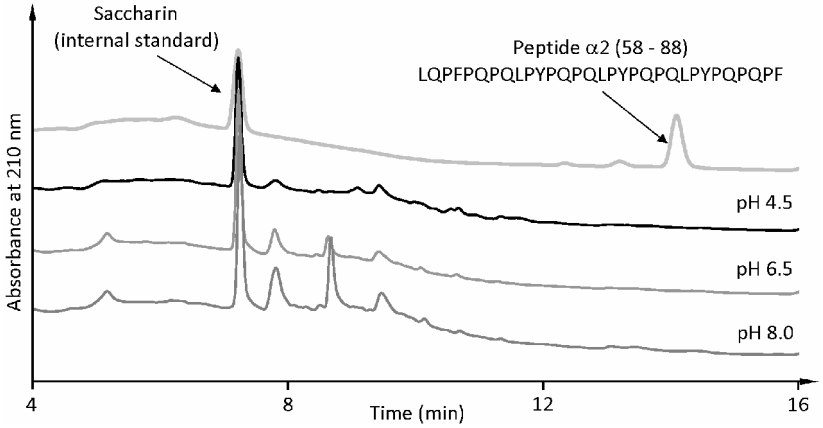

FIG. 1. *Degradation of the coeliac active peptide α2 (58 – 88) at different pH values, with a sample enriched in peptidases from germinated rye. Peptidases from 100 mg rye bran were incubated with 50 µg peptides for 4 h at 37 °C*

Conclusion

A series of studies has been conducted on the preparation and application of peptidases capable of degrading gluten and gluten-derived peptides into fragments non- toxic to CD patients. Peptidase sources are bacteria, fungi and germinated cereals. Potential applications can be divided into treatment of gluten-containing food as well as therapeutic use. In principle, gluten-containing raw material and food can be effectively rendered gluten-free by peptidase treatment, which is advantageous in products that do not need gluten functionality for quality parameters. In terms of oral therapy, it should be pointed ou that most CD patients can be cured effectively by means of a gluten-free diet, which has no potential side-effects. Alternative therapies should have a similar safety profile and one must carefully weigh the risks, benefits, and the costs of alternatives. In particular, the issue about whether peptidase samples for oral use should be applied on a regular or occasional basis still needs to be discusssed in depth.

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IV. AOECS

AOECS
Association of European Coeliac Societies*

Hertha Deutsch

AOECS-Codex-Delegate, Vienna, Austria

Introduction

AOECS is the umbrella organisation of national coeliac societies in Europe and is an independent non-profit association. AOECS was founded in 1988 and comprises today 37 coeliac societies from the following 33 countries:

Andorra, Austria, Belgium (2), Bosnia-Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic (2), Denmark, Estonia, Finland, France, Germany, Greece (2), Hungary, Ireland, Italy, Luxembourg, Malta, Montenegro, Netherlands, Norway, Poland, Portugal, Russia/St.Petersburg, Serbia, Slovenia, Slovakia, Spain (2), Sweden, Switzerland, Ukrainia and the United Kingdom.

AOECS works on subjects of international importance regarding coeliac disease and the gluten-free diet, coordinates international activities and matters of common interest of the members, exchanges information among the members, gives any possible advice and assistance to small and recently formed coeliac societies and is lobbying for awareness of the gluten intolerance.

Gluten-free diet – legal improvements

Since 1992 AOECS has the status "Observer" in the world-wide Codex Alimentarius Commission and participated in some Codex Committees and all sessions of the Commission since that time. In various AOECS statements the coeliac disease, the need for correct labelling of foods and the high incidence of the gluten intolerant population was brought to the attention of the governments and food manufacturing companies all over the world. This resulted in the fact, that the awareness of gluten intolerance increased considerably in the general food industry worldwide.

AOECS participated very actively in the elaboration and modification of all world-wide Codex Standards and Guidelines for labelling of foods for normal consumption, genetically modified foods and special dietary foods. Further details of all relevant texts and references were written in the paper "20 years AOECS" published in the

* non-edited version

Proceedings of the 23rd Meeting of the Working Group on Prolamin Analysis and Toxicity from 25-27 September 2008. As usual Codex Standards are transferred into national food legislation and these improvements have been implemented in national food legislation e.g. EU-Directives.

With the adoption of the "Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten" in July 2008 and the equivalent "Commission Regulation (EC) No 41/2009" of January 2009 all legal requirements for a save gluten-free diet were established and the most important aim for coeliacs fulfilled.

International Gluten-free Symbol

The "Crossed-Grain-Symbol", known as the "Gluten-free Symbol" has been used on special dietary products and information material about coeliac disease since more than 40 years. The owner of this symbol is Coeliac UK who gave permission for usage to national coeliac societies. Because of different national interpretations about the term "gluten-free" the meaning of the Gluten-free Symbol was very varying within Europe. The AOECS Working Group Codex, Labelling and Symbol elaborated the "European Licensing-System" which was adopted by the AOECS General Assembly in September 2009.

European Licensing System

The benefit of the European Licensing System is the harmonisation for the usage of the Crossed-Grain-Symbol in all countries within and outside Europe. It consists of the AOECS Standard, the AOECS Charta and the Licensing Contract.

AOECS Standard

The "AOECS Standard for Foods for Persons Intolerant to Gluten" is based on the worldwide "Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten" and is an integral part of the License Contract. It describes the food groups which are permitted bearing the Symbol, specifies technical requirements, determines

the thresholds, the analytical method, the frequency of analytical testing and has six Annexes:

Annex I: List of food products which are not permitted bearing the Crossed Grain Symbol because these products are always gluten-free e.g. fruits, milk etc.

Annex II: HACCP Guidance for flour, flour mixture and mill

Annex III: HACCP Guidance for bakery products

Annex IV: HACCP Guidance for pasta production

Annex V: HACCP Guidance for foods for general consumption

Annex VI: HACCP Guidance for confectionary products.

AOECS Charta

The AOECS Charta is the contract between AOECS and its member associations to guarantee a unified European Licensing System of the Crossed Grain Symbol on food products in the AOECS Territory. The Charta regulates all necessary procedures e.g. certification guidance, license fees, administration, monitoring and the registration numbering system.

License Contract

The License Contract is the agreement between the food producer and the national coeliac society. It guarantees that coeliacs can rely that products bearing the Crossed-Grain-Symbol with the registration number below fulfil the same technical requirements in all countries in terms of avoiding contamination controlled by the same analytical testing which is for the time being the R5-method.

Development in CCMAS

In March 2010 the day before the plenary session of CCMAS (Codex Committee on Methods of Analysis and Sampling) a workshop was organised by IAM/MoniQA where the item "Proprietary Methods" was discussed among other subjects. Although "Proprietary Methods" is a general issue, special attention was given to the R5-method. A lot of concerns were raised by some delegates, e.g.: regarding availability of reagents; restricted licensing of antibodies; the danger that the adoption of a proprietary method created an anti-competitive situation; methods should have an independent validation, not manufacturer's validation; to describe the method more generically for the purpose of use as a Codex method etc.

AOECS explained the difference between the Skerritt and the R5-method and draw the attention of the delegates to the result if different methods will be adopted by Codex: we know from several studies that the same food sample tested with different antibodies, standards and methods give extremely varying results. The consequence for the European Alert System and food labelling legislation has to be considered if nobody knows which test is right and which is wrong. AOECS strongly recommended that an independent scientist or small group of scientists, who have outstanding knowledge about gluten analysis, should make a ring test comparing well known and new methods aiming to suggest the best method from the scientific point of view.

Further on AOECS recalled that because of the request of Codex the Prolamin Working Group was established more than 20 years ago to advise Codex regarding gluten analysis. Few years ago the Prolamin Working Group recommended the R5 method.

At the plenary session of CCMAS at the agenda Nr. 8 "Report of an interacengy meeting on methods on analysis and sampling" this item was discussed. The IAM had prepared a first draft paper, presented in the Annex to CRD 2 for information. The paper noted that proprietary methods were not clearly defined, highlighted some concerns that could arise from their use: they might prevent further development of new and better techniques, distort competition between companies producing the reagents, and create difficulties for government authorities if particular reagents were not readily available for official methods. It was recalled that the R5 method for the determination of gluten illustrated some of these problems as the reagents were not generally available. Several approaches were proposed in CRD 2 to address this issue, including the use of the criteria approach in Codex.

The Delegation of New Zealand recalled that it had earlier proposed to put forward a new procedure for evaluation of methods, and offered to contribute to future discussions on this issue.

AOECS contributes in the discussion. The statement is included in the CCMAS report as follows:

"The Observer from AOECS recalled that the R5 method is the most accurate method from the scientific point of view for the time being, and that if different methods were allowed, it would create serious problems as to how to handle different results for the same food sample: if one method detects a gluten content higher than 20 mg/kg gluten and another method detects a level below 20 mg/kg, it cannot be determined whether the food can be labelled "gluten free" or not. Moreover, the Observer noted that a method which underestimates the gluten content in foods poses severe health risks for gluten intolerant-consumers."

It was noted that the IAM would proceed with its consideration of proprietary methods, invited wider contribution than only IAM members and would provide an update to the next session of the Committee.

AOECS kindly asks the Prolamin Working Group to consider this new development in CCMAS and asks very urgent to consider whether the Prolamin Working Group or a group member could take over the very important work to compare all existing gluten analysis to give the best possible advise to Codex in this extremely important issue for the consumers health, the food industry and national food authorities.

V. Perspectives and action plan

Perspectives and action plan

Martin Stern

University Children's Hospital, Tuebingen, Germany

The Prolamin Working Group executive meeting and joint discussion held on October 1, 2010, led to the decisions outlined below.

Action plan

I. Analytical

- PWG gliadin is available in 100 mg batches from the PWG chairman (martin.stern@med.uni-tuebingen.de).
- R5 ELISA for gluten analysis based on PWG gliadin continues to be the accepted basis for gluten analysis. New test systems will have to be tested by ring trial.

II. Clinical

- Non-coeliac gluten sensitivity will be included in the spectrum of group activities.
- The relevance of gluten und modified gluten in food technology will be a further focus.

III. Publication and policy

- The PWG home page (http://www.wgpat.com.ar) will be extended to include photographs and research profiles of group members as well as table of contents of the most recent meeting.
- New group members from science and industry will be searched actively.
- The programme for the group meeting in Stuttgart-Fellbach 2011 will be published already in April 2011 (printed flyer in collaboration with Deutsche Zöliakie-Gesellschaft e. V. (DZG).
- This printed, citable book version of the 2010 protocol (print run: 500 copies, with ISBN) was made possible through funding by Dr. SCHÄR GmbH/Srl, Burgstall BZ, Italy), and by the efforts of the production team of Verlag Wissenschaftliche Scripten, Auerbach, Germany. It will be distributed among leaders of opinion in gluten analysis and clinical medicine.

Next meeting: 2011

We are very happy to announce the following venue for 2011:

Stuttgart-Fellbach, Germany

Host:
Sophia Beisel, Dipl. oec. troph.
Judith Suck, B.A.

Deutsche Zöliakie-Gesellschaft e.V. (DZG)
Kupferstr. 36, 70565 Stuttgart, Germany

Phone +49 (0)711 459981-13
Telefax +49 (0)711 459981-50
Email: Judith.suck@dzg-online.de
http://www.dzg-online.de

Time : September 29 - October 2, 2011

Focus of the meeting:

- Gluten in food technology
- Progress in gluten analysis

The meeting will be limited to 45 participants and attendance is by invitation only. Invitations will be dispatched by May 2011.

For registration please contact:
Sofia Beisel, Dipl. oec. troph.
Judith Suck, B.A.
(address: see above)

Very special thanks to the host of this kind invitation!